Chemometric Optimization and Kinetic Developments in Flow Injection Analysis

BY

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بسم الله الرحمن الرحيم

To my beloved parents,

Arafa and Osman

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Chemometric optimization methodologies have been utilized for the development of quantitative methods of analysis of some drugs using flow injection (FI) techniques. Super modified simplex procedures together with statistical design techniques have been used for optimization and modeling of response surface. The sequential optimization has validated the flow injection methods proposed with excellent reproducibility, high sensitivity, and a wide dynamic range. A sampling frequency of not less than 120 h^{-1} was obtained for all methods developed.

Sequential injection (SI) technique has been successfully applied for the determination of reaction stoichiometry and formation constant. The 1 : 1 and 1 : 2 metal : drug mole ratio have been obtained for the complexation of some phenothiazines with palladium(II) in hydrochloric acid media and for the complexation of some flouroquinolones with iron(III) in sulfuric acid media respectively.

For the first time, sequential injection technique has been employed for full kinetics investigation resulting in proposing reasonable mechanism of reactions and adopting kinetic methods of analysis. The complexation reaction of bromazepam with iron(II) was studied in hydrochloric acid media. The orders of the reaction with respect to each reactant were determined and a consistent reaction rate law was offered.

خلاصة الرسالة

إسم الطالب : فخر الدين عثمان سليمان

عنوان الدراسة : **الطرق الإحصائية الكيميائية والتطورات في الكيمياء الحركية في التحليل بالحقن الإنسيابى**

التخصص : كيمياء تحليلية

تاريخ الشهادة : يونيو ١٩٩٦ م

أستخدمت الطرق الإحصائية الكيميائية لتحديد أمثل الظروف بغرض تطوير طرق تحليلية كمية لبعض الأدوية بإستخدام الحقن الإنسيابى .كذلك تم تطبيق طريقة الإفراد المعدلة بالإضافة إلى التصاميم الإحصائية والتشكيل السطحي للإستجابة لتحديد أمثل الظروف للتحليل الكمي . أتبعت هذه الطرق بشكل تتابعى مما أدى إلى ثبوت طرق الحقن الإنسيابى التى تم عرضها ، وذلك بالحصول على درجة إمكانية إستعادة نتائج ممتازة ، وحساسية عالية لهذه الطرق . وقد كان معدل عدد العينات التى تم تحليلها في الساعة الواحدة قد تخطى الـ ١٢٠ عينة بإستخدام الطرق المطورة في هذه الدراسة .

أيضاً تم إستخدام طريقة الحقن التتابعى ولأول مرة بنجاح تام لتحديد درجة الإتحاد العنصري وثابت التكوين بالنسبة لبعض التفاعلات التى درست ، ووجد أن درجة الإتحاد العنصري هي ١ : ١ بالنسبة إلى تراكب الفينوزيازين مع عنصر البلاديوم في محلول حمض الهيدروكلوريك ، بينما وجد أن درجة الإتحاد العنصري لتراكب الفلوروكويولين مع عنصر الحديد هي ٢ : ١ .

تم أيضاً ولأول مرة إستخدام طريقة الحقن التتابعى لدراسة حركية التفاعلات بالتفصيل وذلك بغرض إقتراح آلية مناسبة لمثل هذه التفاعلات بالإضافة إلى تبنى طرق تحليل كميائى حركى . للوصول إلى هذه الأهداف أستخدم التفاعل التراكبى بين البرومازيبام والحديد(II) من حمض الهيدروكلوريك المخفف . تم بنجاح في هذه الدراسة تحديد رتبة التفاعل لكل المواد الداخلة في التفاعل كما تم عرض معادلة حركية ملائمة ومن ثم طريقة مثلى لتحديد تركيز مادة البرومازيبام من المستحضرات الطبية .

درجة الدكتوراة في العلوم

جامعة الملك فهد للبترول والمعادن

الظهران ، المملكة العربية السعودية

يونيو ١٩٩٦م

PUBLICATIONS

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CHAPTER ONE

1. INTRODUCTION

1.1 Objective

The principal objectives of the present research work are :

1. To apply chemometric optimization techniques to flow injection analysis (FIA), either to improve already existing methods or to alternatively propose a better method of analysis for the determination of a selected example of some important drug families.
2. To apply statistical experimental design in conjunction with response surface modeling in an attempt to qualitatively understand the behavior of the analytical system and also to correlate this behavior with the kinetics of the chemical reaction.
3. To exploite the sequential injection analysis as (i) a tool for kinetic determination of drugs in pharmaceutical products, (ii) a vehicle to monitor chemical kinetics and to use the kinetic effects to elucidate a plausible reaction mechanism and (iii) to determine reaction rate constants whenever possible.
4. To apply the sequential injection analysis to some fundamental studies, for example, to determine the stoichiometry of the reaction, equilibrium constants.

1.2 Present Status of the Problem

It is well known that in flow injection analysis two distinct processes take place simultaneously, physical dispersion of the sample zone within the carrier stream of the reagent and a chemical reaction between the analyte and the reagent. When the detector is tuned to sense species produced by this chemical derivatization, the readout has the form of a peak which is now termed as fiagram[1] . Since its birth in the early 1970's, flow injection analysis (FIA) has been demonstrated as a tool for serial assays with excellent reproducibility, low consumption of sample and reagent per assay, and a high sampling frequency.

The FI apparatus, in its simplest form, is composed mainly of a pump by means of which a carrier stream is propelled through a narrow tube, an injection port which is used to inject a well-defined zone of sample solution into the carrier solution; and a reactor. Typically, the reactor is a narrow tube that has been coiled to enhance radial mixing of sample and reagent. If several reagents must be added in succession, additional streams are confluenced and coils are added.

Optimization of a flow injection system, considered the chief objective of this work, is meant to compromise dispersion of the sample zone, its mixing with a reagent, and the time required to achieve the desired chemical conversion of an analyte into a detectable species, in order to minimize undesired zone broadening and to maximize sensitivity and sampling frequency.

Results of studies of the influence of experimental variables on the analytical signal (S) are usually used to choose the optimum operating conditions. Determination of the optimum operating conditions is considered the corner stone in the development and characterization of an analytical method. A single factor at a time approach , surprisingly, is a widely and extensively used method of optimization until now. In this approach the analytical signal (S) is expressed graphically as S vs. the variable, by keeping all other variables constant, and the level of the variable at which the signal is maximum is considered the optimum level of that variable. The single-factor-at a time approach does not always yield the optimum conditions, especially when the experimental variables interact with each other. Alternatively, in the simplex optimization approach[2,3], which is a multivariate optimization technique, several experimental variables are changed simultaneously and a systematic search for the optimum response is made. The simplex is a geometric figure whose vertices are n+1, where n is the number of variables. The simplex is moved across the response surface by a prescribed set of rules until it reaches the optimum response or fails. The modified simplex adjusts to the error surface by expansion or contraction resulting in faster convergence. Most of the recent modifications and developments of the simplex algorithm have been mainly focused on improving the convergence of this optimization technique on complex surfaces.[4,5] The chances of finding a global solution to simplex optimization may be enhanced by application of a second optimization technique prior to the simplex search. The employment of the simplex method to characterize an analytical method for the

determination of drug analytes in complex pharmaceutical preparations by flow injection (FI) was presented by Sultan.[6-8] In the methods described the modified simplex method was utilized in the selection of the proper experimental conditions for the flow injection method, resulting in improved figures of merit and high sampling frequency.

Statistical experimental design has become one of the most widely used optimization tools in the chemical literature during the past decade. A factorial design was used to study the effects of flow rate, injection volume, and coil length on the experimental fluctuations of flow injection signals[9]. An analysis of the fluctuations revealed that interaction between parameters was always significant. A factorial design strategy was employed for the optimization of HPLC[10]. The optimization of TLC separations of alkaloids was approached with a mixture design and response surface modeling[11]. The number of mobile-phase solvents used for the separation was reduced from eight, recommended by the European Pharmacopoeia, to four without a loss in resolution.

Optimization methods can be used in conjunction with an experimental design strategy. The initial experimental design step can often improve the chances for good solution by providing more information about the response surface. A coupled strategy based on experimental design and simplex optimization was used to optimize the supercritical fluid extraction of polyaromatic hydrocarbons(PAHs) and organochlorine pesticide compounds from the environmental samples using a liquid-solid extraction cartridge[12]. Also simplex optimization has been applied after factorial designs in atomic

spectroscopy[13] and HPLC[14]. However, besides the optimization, statistical experimental designs coupled to optimization procedures can be used to model the response surface. This would clearly lead to better understanding of the nature of the chemical and physical behavior of the system.

The choice of a specific analytical method for a given analyte in a particular sample depends on the relative importance of the evaluation criteria and figures of merit to the specific situation expected[15]. This choice, for analytes in drug formulations, rely on a number of requirements including the accuracy, precision, analysis time, as well as the range of concentrations of analyte and concomitants in the sample matrix. Also factors such as the budget, type of personnel involved and amount of initial sample available should be included in the list of requirements for this choice.

Ideally, an analytical method should be rugged with respect to uncontrolled changes in the experimental variables. Critical variables which cause large changes in response when their levels are varied should be adjusted carefully to improve precision and accuracy of the method. Also the speed of the analytical method can be improved by controlling only those factors which have a great effect on the response without sacrificing the precision or accuracy of the method. Factor tolerance is important not only to obtain good precision, but also to avoid wasting resources on unnecessary fine control of insignificant factors[16]

Slowness of some of the reactions investigated deemed necessary a study of these reactions using kinetic methods of analysis. Kinetic methods

of analysis are based on the kinetics of chemical reactions and they make use of the response of a dynamic system to assay the analytes in their respective samples. The advantages of analytical methods based on dynamic systems over those based on traditional equilibrium systems can be found elsewhere.[17,18] Most of the criticism about kinetic methods is due to the lack of accurate reproduction of the reaction conditions in each experimental determination. Reproducibility in the experimental conditions is therefore a major concern in kinetic methods compared to equilibrium methods especially regarding the time which is more critical in the former.[17] From the foregoing it can be concluded that the major requirements of a successful kinetic method of analysis are, accurate timing, careful adjustment of experimental conditions, precise sample and reagent measurements and accurate measurement of the response signal.

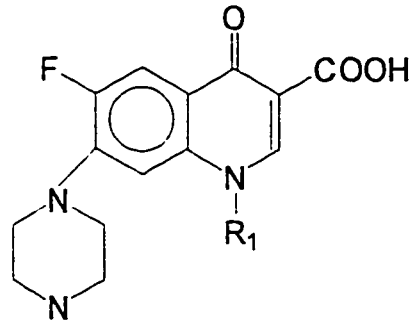
Sequential injection analysis (SIA) which is considered as the secondary objective to be tackled in this work was introduced by Ruziüka and Marshall[19] as a second generation of stopped-flow technique is based on the sequential aspiration of a sample and reagent through a selector valve into a holding coil to form a stack of a well defined zones. When the direction of the flow is reversed a composite zone in which sample and reagent zones penetrate each other is formed and injected into a reactor and then into a detector. For kinetic determinations the flow can be stopped and a portion of the composite zone is then trapped inside the flow cell of the detector for reaction rate measurements. This technique is very promising for kinetic determinations because injection volumes, reaction times and zone

dispersion can all be changed readily and precisely by varying sequenced volumes, flow rate, stopped-flow times and reversals via computer control of the pump[20]. The technique is still in its infancy, and has not been well utilized especially for kinetics methods of analysis and therefore it should be exploited extensively in this field. Application of SIA to kinetic methods of analysis, if successfully accomplished, will be a potential introduced for the first time in literature.

1.3 Drug Families Studied

1.3.1 Fluoroquinolones:

The history of the 4-quinolones starts with the discovery of nalidixic acid in the early 1960's. [21] In the early 1980's, modern fluoroquinolones, such as ciprofloxacin, norfloxacin and flumequine, were introduced. The main difference between the classical quinolones and the fluoroquinolones is that the latter contains a fluorine atom at the C-6 position and a piperazine derivative group at the C-7 position (Scheme 1.1). It has been shown that the fluorine atom enhances the DNA-gyrase complex binding and cell penetration.[22] The introduction of the piparzine moiety increases antibacterial potency far greater than other common antibiotics like tetracyclines. The fluoroquinolone antibiotics are active against 37 different kinds of bacteria. They have been recommended for the treatment of urinary tract infections, bacterial prostatis, sexually transmitted diseases, gastrointestinal infections, respiratory tract infections, bone and joint



(1) Ciprofloxacin

R_1 = 

(2) Norfloxacin

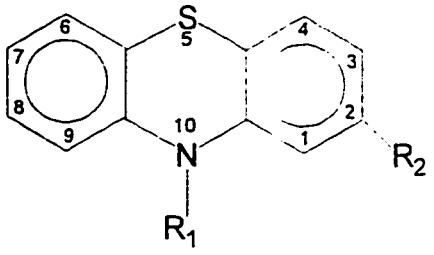
$R_1 = C_2H_5$

Scheme 1 . 1 Chemical structure of fluoroquinolones

infections, skin and soft tissue infections and many other related infections.(23)

1.3.2 Penothiazines

Phenothiazine is a three-ring structure in which two benzene rings are attached to each other by a sulfur and a nitrogen atom in the C-5 and the C-10 positions, respectively (Scheme 1.2). Substitutions usually occur at the 2 and 10 position. Substitution of a chlorine, methoxy, thiomethyl, acetyl or trifluoromethyl group in the 2 position increases the antipsychotic potency of these drugs. The nature of the side chain substituent at the 10 position influences both the potency and the pharmacological activity of the compound. Antipsycotic properties are only present if there are three carbon atoms between the nitrogen in the 10 position of the phenothiazine nucleus and the amine group of the side chain. The presence of a fourth carbon atom in the chain causes a loss of antipsychotic effect of the compound. Reduction of the side chain to two carbon atoms can change the antipsychotic activity to antihistaminic or antiparksonian, depending on the nature of the substituents on the basic amino group. Antipsychotic phenothiazines are divided into three groups based on the nature of the side chain attached to the 10-position: dimethylaminoalkyls, piperazinyl-alkyls and piperidyl-alkyls. The dimethyl amino derivatives, of which chlorpromazine is the best known example, are characterized by significant sedative properties making them useful in the treatment of psychotic patients experiencing sleep disturbances. The piperazine derivatives are the most



Phenothiazine nucleus

(1) Chlorpromazine

$R_1 = CH_2\text{-}CH_2\text{-}CH_2\text{-}N(CH_3)(CH_3)$ $\qquad R_2 = Cl$

(2) Promethazine

$R_1 = CH_2\text{-}CH(N(CH_3)_2)\text{-}CH_3$ $\qquad R_2 = Cl$

(3) Perphenazine

$R_1 = CH_2\text{-}CH_2\text{-}CH_2\text{-}N$(piperazine)$\text{-}N\text{-}CH_2\text{-}CH_2\text{-}OH$ $\qquad R_2 = Cl$

(4) Trimeprazine

$R_1 = CH_2\text{-}CH(CH_3)\text{-}CH_2N(CH_3)_2$ $\qquad R_2 = H$

Scheme 1 . 2 Chemical structure of phenothiazines.

potent antipsychotic compounds and possess less sedative action than the aminoalkyl phenothiazines an example of this group is perphenazine.(24)

1.3.3 β-Blockers

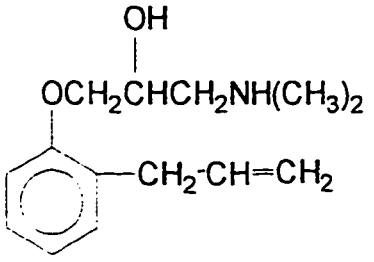
Since their introduction in 1966, β-blocking agents have become the most commonly used drugs for a variety of cardiovascular diseases.(25) The mechanism of blood pressure reduction with β-Blockers remains controversial. One concept is that blood pressure reduction is due to suppression of release of rennin. Another suggestion is that β-Blockers result in a fall of the cardiac output.

β-Blocking agents are competitive inhibitors of the effect of catocholamines at β-adrenergic sites. These drugs are prescribed frequently for the treatment of many diseases, such as hypertension, anxiety, convulsions, anginal effects, disfunctional labor, and migraine. Scheme 1.3 shows two examples of this family of drugs, namely procanamide and oxprenolol.

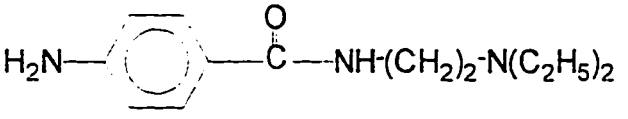
1.3.4 Benzodiazapenes

Benzodiazapenes were synthesized some forty years ago by treating quinazolines, six-membered ring structures, with various amines. When one of the products was investigated later, it was found to have an

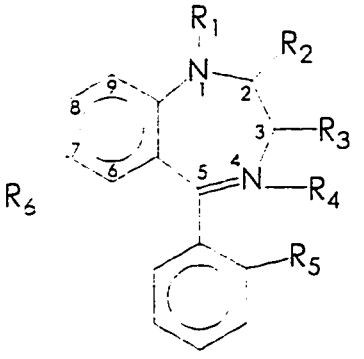
(1) Oxprenolol



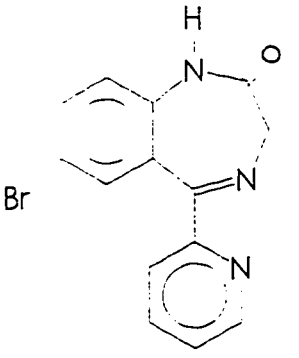
(2) Procanamide



Scheme 1 . 3 Chemical structure of β-blockers.



1, 4 Benzodiazapines



Bromazepam

Scheme 1 . 4 Chemical structure of benzodiazapines and bromazepam.

unexpected seven-membered ring structure and equally unexpected pharmacological activities. Scheme 1.4 shows the general structure of themost active benzodiazapenes together with some examples of this family. The 2-methyl amino group is the most effective substitution at C-2. Activity is reduced when either no group or longer chains are attached to this carbon. An electronegative group at the 7 position is important for antianxiety efficacy and a decrease in potency results from a substitution at the 3 position. A methyl group on nitrogen 1 is optimal as is either no substitution or a methyl group at C-3. These compounds are relatively insoluble in water.(24)

1.4 Theoretical Background

1.4.1 Chemometric Optimization

The basic purpose of providing quantitative analytical data on the composition of a material is to relate some desired property of the material to its composition. The desired property is usually related to variables of composition by some specific functional mathematical form which may be known or unknown in part with empirical components. A host of statistical and mathematical techniques is usually employed to derive the relationship between the desired property and the variables of composition and to account for the uncertainty in the corresponding mathematical model of interaction.

Of an identical nature is the evaluation of a chemical analysis system which produces the quantitative data on a given material. Variables which affect the result interact in an unknown mathematical functional form and a

variety of similar techniques are used to provide insight into the physical and chemical behavior of the measurement system that is used. Statistical and other mathematical techniques are used to determine the importance of certain variables on the end results.

The optimization of chemical systems using mathematical methods has become widespread in the chemical sciences. The early, widespread use of the simplex optimization in chemistry can be explained by its simplicity and its relatively modest demands on the computer as compared to many other nonlinear optimization methods. Even though modern day desktop computers offer substantially more power than earlier machines, the simplex method continues to be very popular among chemists.

In order to apply the mathematical results and numerical techniques of optimization to concrete chemical problems, it is necessary to clearly delineate the boundaries of the system to be optimized, to define the quantitative criterion on the basis of which candidates will be ranked to determine the best, to select the system variable that will be used to characterize or identify candidates, and to define a model that will express the manner in which the variables are related.

The key element in formulating a problem for optimization is the selection of independent variables that are adequate to characterize the operating conditions of the system. It is important to differentiate between system variable that can be treated as fixed and those that have great influence on the response.

Before undertaking any optimization study, it is important to clearly define the boundaries of the system under investigation. The variables

included in an optimization protocol are usually bound between an upper and a lower levels. The variables are prohibited from being smaller and larger than given threshold values. The threshold values could either be determined by the investigator(e.g. a reagent concentration beyond which the response is very undesirable), or by the physical limitations of the instrument used. Given that we have selected the system of interest and have defined its boundaries, we next need to select a criterion on the basis of which the performance or design of the system can be identified. In many chemical analysis optimizations, only one criterion or performance measure is usually used to define the optimum. In most of the cases, the choice of such criteria is limited to the sensitivity. However, there are considerable other choices which are sometimes as important as the sensitivity, such as the time of analysis, reproducibility, etc. It is not possible to find a solution that, for example, simultaneously maximizes the sensitivity and minimizes the time of analysis. Because in many practical situations, it would be desirable to achieve a solution that is best with respect to a number of different criteria. Therefore, it is necessary to formulate the optimization problem with a single performance criterion. Another way of treating multiple competing objectives is to select one criterion as primary and the remaining criterion as secondary. The primary criterion is then used as an optimization performance measure while the secondary criteria are assigned acceptable minimum or maximum values and are treated as problem constraints.

When the performance criterion, boundary conditions and the independent variables have been selected, the next step is to assemble the model that describes the manner in which the problem variables are related

and the way in which the performance criterion is influenced by the independent variable. In principle, optimization of chemical systems may be performed directly, since the mathematical model that relates the independent variables to the performance criterion is unknown. Thus, the independent variables of the system may be set to selected values, the system operated under those conditions, and the system performance index is evaluated using the observed performance. The optimization methodology would then be used to predict improved choices of the independent variable values, and the experiments continued in this fashion.

1.4.1.1 Simplex Techniques

The simplex technique was first introduced by Spendley et al.[2] and later modified by Nelder and Mead[3] and Routh et al.[26] Deming and Long[27] were the first who proposed and developed the method for chemical analysis problems.

A simplex is a geometric figure whose vertexes equal to one more than the number of the dimensions in the factor space. For instance, a simplex in two dimensions is a triangle and in three dimensions is a tetrahedron. When using the simplex for optimization of experimental systems, each vertex corresponds to a set of experimental conditions (coordinates). The responses obtained for each experimental conditions are evaluated in such a way that the parameter setting that results might yield a better response. The response at a certain vertex can be defined as the result of one or more experiments carried out with the parameter settings corresponding with the coordinates of the

vertex. The simplex moves by replacing the vertex yielding the worst response by an estimated better vertex. This is the main concept of all simplex methods, and the difference between the basic simplex, [2] the modified simplex, [3] and the super modified simplex, [26] lies in the way the better vertex is determined.

1.4.1.1.1 Basic Simplex Algorithm

In this algorithm, the initial simplex, termed also as the start simplex, contains n+1 vertexes where n is the number of experimental factors included in the optimization. The coordinates of these vertexes are calculated by the method of Spendley et. al.[2] The responses at the vertexes are then sorted out and the vertex with the worst response (W) is rejected and replaced by a new vertex at point (R)(see Fig. 1.1). This point is obtained by reflecting the worst vertex through the face connecting the best vertex (B) and the next to worst vertex (N). The coordinates of the new vertex are calculated using the following equation

$$V_{x,j} = Y.V_{w',j} + (1 - Y).V_{w,j} \qquad 1.1$$

where $V_{w',j}$ is the average of all vertexes of the simplex except the vertex with the worst response (V_w); it is also known as the centroid of the simplex. Y is the contraction /expansion factor. In the basic simplex algorithm Y is a constant value which is usually set equal to 2. The procedure is continued by replacing the vertex with the worst response by the reflected vertex and

finally halted when the stop criterion is satisfied. The most popular stop criterion is that if the difference between each individual response and the mean response in a given simplex fall below a predetermined value then the procedure is halted.

The simplex technique usually requires considerably fewer experiments than other simultaneous techniques. The main disadvantage of the original simplex algorithm is that a local optimum may be encountered rather than a global one, also very sharp optimum might escape detection. The modified and super modified simplex methods[2,3] were actually developed to overcome these problems.

1.4.1.1.2 The Modified Simplex Method

Nelder and Mead[3] introduced two major modifications to the original simplex . These modifications allow the simplex to expand in the direction of favorable responses and contract in the direction of unfavorable responses. The two basic modifications introduced into the basic simplex algorithm are as follows:

1. If the response of the reflected vertex is better than the response at the best vertex in the current simplex($Rr>R_b$) then an expansion is considered. The expanded vertex is calculated by substituting Y = 3 in equation (1.1). The response at the expanded vertex is evaluated. The expanded vertex is accepted as the new vertex if the response obtained by the parameter settings is greater or equal to the response at the reflected vertex, otherwise the reflected vertex is accepted as the new vertex.

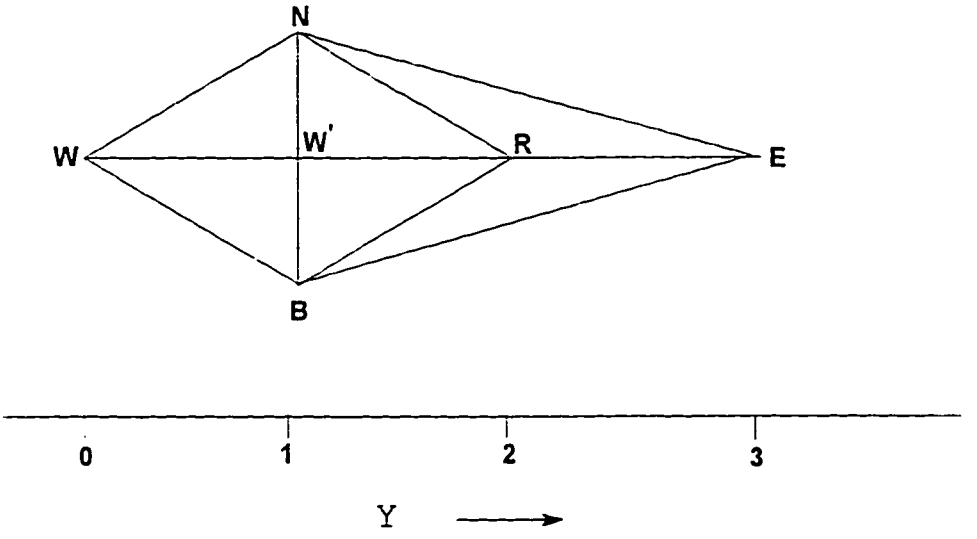


Figure 1 . 1 Representaion of a simplex in two dimensions. W, vertex with worst response; B vertex with the best response; N; vertex with the next to worst response; W', centroid; R reflected vertex; E, expanded vertex;

2. If the response at the reflected vertex is less than the response at the next-to worst reponse in the current simplex a contraction is considered. Two contractions are possible:

a) If the response at the reflected vertex is equal to or better than the response at the worst vertex in the current simplex ($R_r \geq R_w$), then the contracted vertex is calculated by substituting Y = 1.5 in equation (1.1).

b) If the response at the reflected vertex is worse than the response at the worst vertex in the current simplex ($R_r < R_w$), then the contracted vertex is calculated using Y = 0.5 in equation (1).

In the original simplex algorithm[2] the contraction situation is known as the reduction step and is performed by replacing all the vertexes of the current simplex by a set of new vertexes. This set of new vertexes is calculated by subtracting the coordinates of each of the vertexes of the simplex from those of the vertex with the best response and then dividing by 2. In this case, the n+1 experiments should be conducted at each reduction step compared to only one experiment in the case of the modified simplex.

1.4.1.1.3 Super Modified Simplex

In the super-modified simplex algorithm[26] the differences in responses at the simplex vertexes are used to determine the vertex with a better response. When the vertex with the worst response is reflected through the centroid of the simplex three vertexes are located on a straight line, these are V_w, $V_{w'}$, and V_r (Fig.1.1). When contraction or expansion is performed

the new vertex will be on the same line with these vertexes. Therefore measuring or estimating the response at the centroid results in three responses; R_w, $R_{w'}$, and R_r. An estimation of the position of the next vertex, which may give a better response, is found by fitting a second order polynomial model or a Gaussian model through these three responses. The position of the maximum of the fitted function decides the position of the new vertex. It is clear that the Y-factor obtained by this procedure is not constant as is the case for the other simplex procedures. This usually results in a more efficient and faster optimization with considerably less number of experiments.

1.4.1.2 Statistical Experimental Design

1.4.1.2.1 Factorial Designs

Factorial designs are widely used in experiments where several factors are involved and it is necessary to study the joint effect of these factors on a response. By a factorial design we mean that in each complete trial or replication of the experiment all possible combinations of the levels of the factors are investigated. The effect of a factor can be defined as the change in response produced by a change in the level of the factor. This is usually termed as the main effect because it refers to the primary factors of interest in the experiment. The main effect of a factor could be thought of as representing the difference between the average response at the first level of the factor and the average response at the second level of that factor. The term interaction effect is used when the difference in response between the

levels of one factor is not the same at all levels of the other factors. The most elementary class of factorial experiments is the 2^k factorial, that is, k factors each is taken at two levels. The 2^k design is particularly useful in the early stages of the experimental work, where there are likely to be many factors investigated. It provides the smallest number of experiments with which k-factors can be studied at all possible level combinations. Because only two levels for each factor are involved, we must assume the response to be approximately linear over the range of the factor levels chosen.

When one fits a first order equation to a 2^k factorial experiment, it is often convenient to code the independent variables, with -1 representing the low level of a variable and +1 the high level. As an example consider a 2^3 factorial design, the design matrix (in terms of coded variables) together with the treatment combination designation in the margin of the matrix (the presence of lower case letter in the notation implies that the factor in question is at the high level) is given below:

$$D = \begin{array}{c} \begin{bmatrix} x_1 & x_2 & x_3 \\ -1 & -1 & -1 \\ 1 & -1 & -1 \\ -1 & 1 & -1 \\ -1 & -1 & 1 \\ 1 & 1 & -1 \\ 1 & -1 & 1 \\ -1 & 1 & 1 \\ 1 & 1 & 1 \end{bmatrix} \end{array} \begin{array}{c} (1) \\ a \\ b \\ c \\ ab \\ ac \\ bc \\ abc \end{array}$$

The treatment combinations are not necessarily run in the order given in this design matrix, rather, the order should be randomized. (28)

1.4.1.2.2 Two-way ANOVA

Analysis of variance (ANOVA) has been defined as a statistical technique for analyzing measurements that depend on several kinds of effects operating simultaneously, in order to decide which effects are important and to estimate these effects. The major objective of analysis of variance is to investigate the effect of the various factors on the variability of data to determine which portion of the variation is due to systematic reasons (factors) and which is due to random effects. In analytical chemistry analysis of variance usually serves:

1. to distinguish between source of variability between laboratories, between samples and between replications.
2. to investigate the influence of factors (human, instrument, or chemical factors) on the results of a chemical analysis.

When applying ANOVA one assumes that the errors are normally distributed, and are statistically independent and having the same variance. ANOVA assumes that the variances of random variables, due to the effect of independent factors, are additive. Therefore, ANOVA can be utilized to break down the total variance into its components, that is, into a sum of several distinct components, each corresponding to a source of variance.

Let us consider two factors A and B which are assumed to influence the response variable y. Factor A has a-levels (a = 1, 2, ..., i) and factor B is at b-levels (b = 1, 2, ..., j). For each combination of levels an experiment is performed and a response y_{ijk} is obtained by carrying nk observations,

where k represents the replication of each of the n-experiments. The two-way model that can describe such situation is given below:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \tau_{ij} + \varepsilon \qquad 1.2$$

where μ represents an overall mean, α_i represents the effect of the ith level of factor A, β_j represents the effect of the jth level of factor B, τ_{ij} represents the effect of the interaction between factor A and B, and ε_{ijk} is a random error component.

The null hypotheses Ho ($\alpha_i = 0$) (factor A has no significant effect), Ho ($\beta_j = 0$) (factor B has no significant effect), and Ho ($\tau_{ij} = 0$)(there is no interaction effect) is then tested. Statistical tests based on the Fisher F-ratio may then be performed to test the null-hypotheses. These F-tests are determined from the respective component of variance to the component of variance corresponding to the pure experimental error. The calculated F-ratio can then be compared with tabulated F-values using the degrees of freedom corresponding to the numerator (degrees of freedom of the given factor) and the degrees of freedom of the denominator (degrees of freedom of the error). If the calculated F-ratio exceeds the tabulated values at the chosen level of significance, the null hypothesis will be rejected. If on the other hand the calculated value of the F-ratio is smaller than the table value, then the null hypothesis will be accepted and the factor examined does not affect the mean value.

1.4.1.2.3 Response Surface Modeling

Response surface methodology (RSM) is a collection involving experimental strategy, mathematical and statistical techniques, when combined, enable the experimenter to make an efficient empirical exploration of the system in which he is interested. [29]

For example suppose that an experimenter is concerned with a system involving some response ψ which is influenced by a set of independent variable $\zeta_1, \zeta_2, ..., \zeta_k$. The observed response ψ_o as a function of the levels of $\zeta_1, \zeta_2, ..., \zeta_k$ could be written as

$$\psi_o = f(\zeta_1, \zeta_2, ..., \zeta_k) + \varepsilon \qquad 1.3$$

where ε is a random error component. In most of the RSM problems, the form of the relationship between the response and the independent variables is unknown. Thus the first step in RSM is to find a suitable approximation for the true functional relationship between ψ_o and the set of independent variables. Usually, a low-order polynomial in some region of the independent variable is employed. Of course, it is unlikely that a polynomial model will be a reasonable approximation of the true functional relationship over the entire space of the independent variables, but in a relatively small region they work quite well.

The methods of matrix least square methods[29, 30] are usually used to estimate the parameters in the approximating polynomials. The response-surface analysis is then done in terms of the fitted surface. If the fitted surface is an adequate approximation of the true response function, then

analysis of the fitted surface will be approximately equivalent to analysis of the actual system.

An experimental design for fitting a second-order polynomial must have at least three levels of each factor so that the model parameters can be estimated. The experimental design should be chosen on the basis of relative precision in estimating coefficients and the amount of experimental effort required. The 3^k factorial experiment, a factorial experiment with each factor at three levels is usually the choice when the number of factors k is small e.g., 2 or 3. However, when the number of variables under study is greater than 3, the number of observations needed is excessive. A workable alternative to the 3^k factorial is a special type of design called central composite design. The central composite design consists of a 2^k factorial or fractional factorial design augmented by a 2^k axial points and n_O center points. The design matrix for a central composite design where k = 3 is shown below:

$$D = \begin{bmatrix} x_1 & x_2 & x_3 \\ -1 & -1 & -1 \\ -1 & -1 & -1 \\ -1 & 1 & -1 \\ -1 & 1 & 1 \\ 1 & 1 & -1 \\ 1 & -1 & 1 \\ 1 & 1 & -1 \\ 1 & 1 & 1 \end{bmatrix} \begin{bmatrix} 0 & 0 & 0 \\ -\alpha & 0 & 0 \\ \alpha & 0 & 0 \\ 0 & -\alpha & 0 \\ 0 & \alpha & 0 \\ 0 & 0 & -\alpha \\ 0 & 0 & \alpha \end{bmatrix}$$

Where α and $-\alpha$ represents the axial points.

Strategies based on coupling optimization method to experimental design are very helpful in various aspects. A factorial design preceding an optimization technique is very informative, and can help in selecting variables that are most important in order to include in the optimization study. The ruggedness of the analytical method can be inferred from the behavior of the different variables at the levels selected for operation. Response surface modeling improves the understanding of the relationship between the independent variables and the response function, and can also help in relating, qualitatively, this relationship to the kinetics of the chemical system.

1.4.2 Kinetic Development in FIA

In stopped-flow injection the sample is injected into a carrier stream, reagent is added, and the mixture transported into the flow cell where the flow is stopped. Advantages of this method over the continuous flow method are(20, 31):

1. Increase of sensitivity because reactants and products are no longer being diluted, and background signals can be eliminated as they remain unchanged during the stopped-flow interval.
2. Accuracy can be improved as the measurements are less sensitive to perturbations such as lag phases and deviation from linearity.
3. Provide kinetic discrimination and therefore enhance selectivity because kinetic measurements are less subject to interferences.
4. Consumption of reagent solutions is greatly reduced, because reagents are used only when needed rather than continuously.

Gradient stopped-flow FIA[1] is ideally suited for measurement of reaction rates and rate laws of chemical reactions because the FIA peak profile provides as infinite number of reactant ratios, so that stopping the flow at suitable positions on the peak allows the adjustment of conditions to first order, second order, etc. It was found that single line FIA systems provide data that is difficult to interpret[32], because the initial reagent concentrations are not known, because mixing of sample and reagent by mutual penetration begins at the moment of injection and proceeds continuously to the flow cell detector[20, 33]. Conversely, a two-line system was used with ease to measure the rate constants for reactions of permanganate with benzaldehyde and crotonic acid[33].

A general treatment for the determination of rate laws and rate constants will be presented here with the aid of a two-line manifold (Fig. 1.2). Consider the reaction:

$$a\,A + B \rightarrow \text{Products} \qquad 1.4$$

By injection of analyte (A) into a carrier stream (C), a well defined zone is formed which disperses on its way through a reaction coil (RC_1). The concentration of analyte (A) can be obtained from the knowledge of the dispersion coefficient as follows

$$C_A = C_A^o / D_A \qquad 1.5$$

where C_A^0 is the analyte (A) concentration before injection, C_A is the gradient concentration of the zone element and D_A is the dispersion coefficient describing the concentration of C_A within the sample zone gradient element at delay time t_d. When the analyte (A) is injected into an inert carrier stream (C) and the stream is confluenced with reagent (B) in a two-line FIA system (figure 1.2), each element of the dispersed analyte is mixed with the same volume of reagent. The initial reagent concentration C_{Bi} will be well defined as:

$$C_{Bi} = C_B^0 \, Y \, (X + Y)^{-1} \qquad 1.6$$

where X and Y are the pumping rates of the C and B lines respectively. The initial concentrations of analyte C_{Ai} for each delay time are obtained by dispersion experiments in which the horizontal portions of the stopped flow curves correspond to C_A in equation (1.5). Since in coil (RC_2) both chemical reaction and dispersion take place simultaneously, it is necessary to consider the error caused by the reaction of A with R during the time t_m allowed for mixing. If C_{AC} is defined as the concentration of A consumed by reaction in coil (RC_2), the apparent initial concentration of A detected in the flow cell is

$$C_{Ai} = \frac{C_A^0}{D} - C_{AC} \qquad 1.7$$

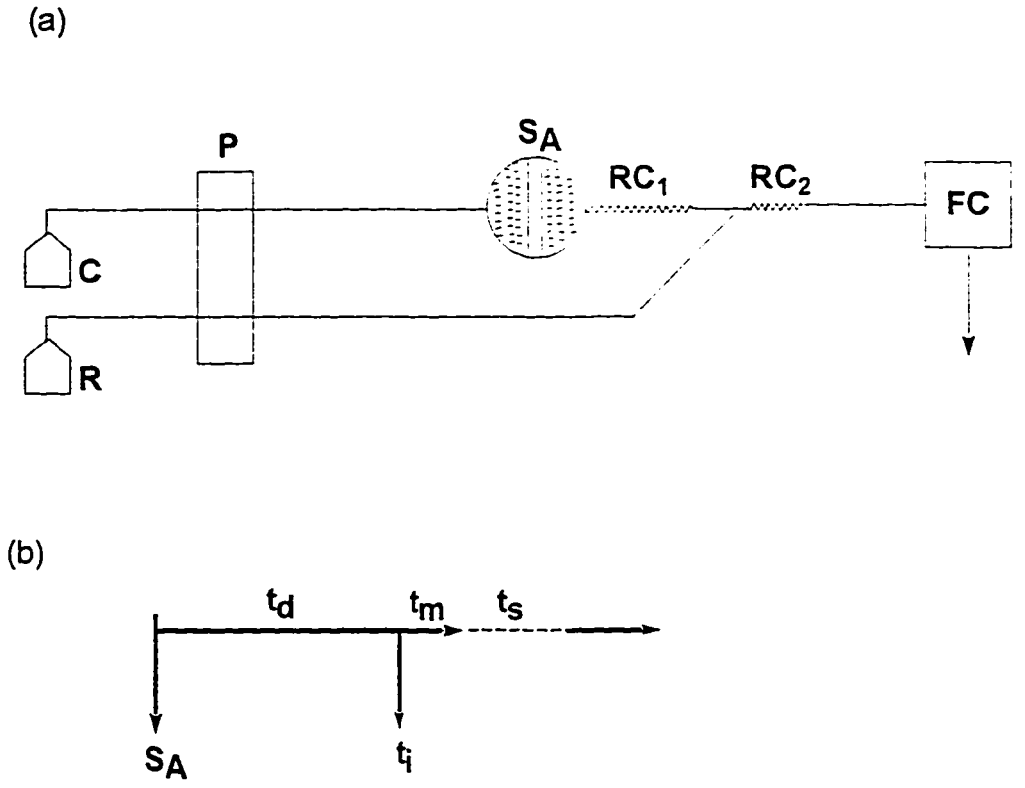


Figure 1 . 2 (a) Double-line FIA manifold for kinetics studies. R, reagent line; C, carrier solution line; P, peristaltic pump; SA, injection valve; RC_1 and RC_2, reaction coils; FC, flow cell. (b) Timing sequences for stopped- flow injection analysis; t_d,delay time; t_i, initial reaction time; tm, mixing time and t_s, is the stopped flow period.

where D is the dispersion calculated at a given delay time, t_d, and C_{AC} is the concentration of A consumed by reaction in coil (RCl). When $t_m \ll t_{1/2}$ of the reaction $C_{AC} \ll C_{Ai}$ then equation (1.7) will reduce to

$$C_{Ai} = \frac{C_A^o}{D} \qquad 1.8$$

The rate law for equation (1.4) is given by

$$\frac{-d\,C_A}{dt} = k_2\, C_{At}\, C_{Bt} \qquad 1.9$$

where k_2 is the second-order rate constant and C_{At} and C_{Bt} are the analyte and reagent concentration respectively at time t initiated at t_i. Integration of this equation will result into

$$k_2\, t = \frac{1}{(a\, C_{Ai} - C_{Bi})} \ln\left[\frac{C_{Bi}(C_{Ai} - x_t)}{C_{Ai}(C_{Bi} - a\, x_t)}\right] \qquad 1.10$$

$$x_t = C_{Ai} - C_{At} \qquad 1.11$$

Substituting equations (1.8) and (1.11) into equation (1.10) and rearranging, we obtain equation (1.12)

$$k_2\, t = \frac{1}{\frac{a\, C_A^o}{D} - C_{Bi}} \ln\left[\frac{C_{Bi}\, C_{At}}{C_A^o/D_A\,(C_{Bi} - a\, x_t)}\right] \qquad 1.12$$

when $C_{Bi} \gg C_{Ai}$ then equation (1.12) will simplify to equation (1.13)

$$\ln \frac{C_A^o}{D} - \ln C_{At} = C_{Bi}\, k_2\, t = k't \qquad 1.13$$

where k' is the pseudo-first order rate constant at a given C_{Bi}. k' can be obtained safely without prior knowledge of C_A^o / D_A, but it has little value if C_{Bi} is unknown. By suitable selection of C_{Bi}/C_{Ai} ratio, pseudo-first order and second order rate constants can be obtained.

FIA kinetic conversion techniques are defined as procedures by which a non-detectable species is converted into a detectable species via a kinetically controlled chemical reaction[34]. Conversion techniques incorporate most of the chemical assays performed under FIA condition, except the cases when the chemical reaction is very rapid and therefore goes to completion before it reaches the detector. A method for the determination of phenolic and hydrazino drugs with 1-fluoro-2,4 dinitrobenzene using a Fluoride-selective electrode was described by Apostolakis et al.[35]. Taking the advantage of the controllable kinetic conditions when executed in FIA the analytes in pharmaceutical products were determined in a micellar medium. Chung and Ingle[36] determined total ascorbic acid using a kinetic fluorimetric method based on a FIA system. In this method, ascorbic acid solutions were injected into a carrier stream of mercury (II) chloride and 1,2-diaminobenzene to form a fluorescent product on line. A fixed-time method was proposed by Sultan for the determination of bromazepam in pharmaceutical preparations[37]. The method was based on stopping the

flow for a given time just after sample injection using single line flow injection manifold.

1.4.3 Sequential Injection Analysis (SIA)

Sequential injection (SI)[19, 20] is the second generation of gradient flow techniques, based on the sequential aspiration of sample and reagent zones through a selector (rather than injection valve) in a channel or a holding coil (figure 1.3). In this manner a stack of a well defined zones is obtained which is then allowed to penetrate each other by flow reversal. The flow reversal creates a composite zone a section of which can be trapped inside the observation zone of the detector, by stopping the flow, for reaction rate measurements. Advantages of SIA[38] include mechanical simplicity, since only a single valve and a single pump is needed, and reliability, because once configured the components and associated flow channel do not need physical restructuring. The degree of zone dispersion can be influenced by tuning the length and the number of flow reversals, and the reaction time can be adjusted through the duration of a stopped flow period.

The measuring cycle of the SI technique comprises the following steps (when a peristaltic pump is used):

(1) Forward pumping of carrier solution with the selector valve in position 1, until the holding coil, reactor and detector have been washed out.

(2) Aspiration of sample solution with the selector valve in position 2 by flow reversal into the holding coil.

(3) Aspiration of reagent solution with the valve in position 3 by another flow reversal.

(4) Forward pumping of carrier solution with the valve in position 1, to propel the composite sample reagent zone through the reactor into the detector.

(5) A stop of the flow when the selected part of the composite zone reach the detector for reaction rate monitoring.

(6) Resumption of the forward flow until all of the reagent and products are washed out of the detector.

Zone sequencing and mutual interdispersion are the key operations of sequential injection analysis (SIA). The dimensions of the holding coil, and of the reactor, the number and duration of flow reversals the volumes of the aspirated zones and the flow rates used, altogether affect the degree of zone overlap within an adequate residence time[39]. In analogy with conventional FIA[31], the dispersion of the sample zone is to be adjusted in order to fulfill the requirements of an intended assay. It was found that this is easily achieved through selection of injection volumes and the delay time at which the forward movement of the zone through the detector is being stopped[39]. For stopped-flow reaction-rate measurement, the choice of delay time(t_d) will allow optimization of the analyte to reagent ratio. Deliberate increase of axial dispersion obtained by flow reversal is required to achieve maximum zone penetration, because in kinetic studies an increase in zone broadening increases the range of useful t_d values at which the stopped flow period may start.

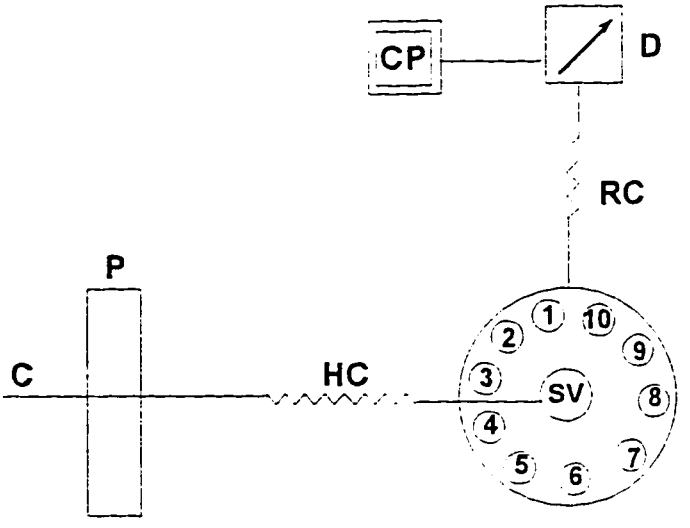


Figure 1 . 3 Typical SI-manifold; C, carrier; P; pump; HC, Holding coil; SV, selector valve; RC, reaction coil; D, detector; CP, computer

1.4.4 Determination of Formation Constants

Most of the methods available for determining formation constants are mainly based on preparing a series of solutions containing known proportions of the complex-forming species, in which the concentration of one of the reactants or products is followed directly or indirectly by a suitable analytical technique.(40)

Many factors, e.g. ionic strength, pH, etc., affect the formation constants. Much more important is that the ligand and the metal ions are the only species involved in complexation. Schwarzenbach(41) introduced the concept of the 'apparent formation constant' in connection with the theory of complexometric titration. Ringbom(42) developed the theory further, and extended the concept to other fields of analytical chemistry. The name 'conditional constant' was introduced by Ringbom is now widely used and recommended, since it well expresses the notion of a term which can be assumed as constant only under given condition.

Introduction of computers has revolutionized the calculation of formation constants from equilibrium data, utilizing modern numerical methods of analysis, making it an easy task for even complicated systems. Many developments in the mathematical algorithms, computer programs and experimental techniques are continuing rapidly to determine formation constants. (43)

1.4.4.1 The Continuous Variation Method

This method was first introduced by Ostromisslensky[44] and later developed by Job[45] to determine stoichiometries and formation constants of complexes. In the method a series of solutions is prepared by mixing different volumes of equimolar solutions of the ligand and the metal ion and diluting to a constant volume to give solutions of the same total molar concentration but different mole fractions. If a single, stable complex is formed, a plot of absorbance versus mole fraction of one of the reacting species gives a characteristic triangular plot. The mole fraction of the maximum of this plot gives the stoichiometry of the complex. Likussar[46] has developed a theoretical approach to the continuous variation method in which no approximations are necessary. The general equations he developed to calculate the formation constant do not contain molar absorbtivities.

Let us consider the general equation for the formation of complex M_mL_n, at equilibrium

$$mM + nL \text{ ----------> } M_mL_n \qquad 1.14$$

where M represents a metal ion, L a ligand, M_mL_n the complex formed in a molar ratio of m:n for M and L. The formation constant K_f for the complexation reaction is then given by the following equation:

$$K_f = \frac{[M_mL_n]}{[M]^m[L]^n}$$

$$= \frac{[M_m L_n]}{\left[\left(C_m - {}^{m}[M_m L_n]\right)^{m}\left(C_L - {}^{n}[M_m L_n]\right)^{n}\right]} \qquad 1.15$$

where C_M and C_L are the total metal and total ligand concentrations respectively. The mole fraction (x) is defined by

$$x = \frac{C_M}{(C_M + C_L)} = \frac{C_M}{k} \qquad 1.16$$

$k = (C_M + C_L)$

utilizing the normalized absorbance concept[47] together with the foregoing equations an expression for calculating the formation constant can be obtained. This expression does not contain molar absorptivity terms and is given by:

$$K_f = [(m+n)/k]^{(m+n-1)} y[(m+n)x - my]^{-m}.[(m+n)(1-x) - ny]^{-n} \qquad 1.17$$

where y is the normalized absorbance.

1.4.4.2 The Molar Ratio Method

This method was introduced by Yoe and Jones[48] and since then it has been extensively used for the determination of stoichiometry and formation constants of complexation reactions. The molar ratio method is based on graphical representation of the observed absorbances versus the molar ratios of the two components of the complex when the concentration of one component is held constant while that of the other component is varied. Momoki et.al[47] developed the theoretical and mathematical algorithm that

describe this method. Equations similar to those described in the continuous variation method are used to develop these mathematical equations which are also free from molar absorbtivity terms. The molar ratio curve is described by the following equation

$$\kappa_M = (m/n).y + (nK)^{-1/m} C_L^{-(m+n-1)/m} . y^{1/m} (1-y)^{-n/m} \qquad 1.18$$

where κ_M is the mole ratio of the metal ion, other symbols are of the same meanings as above.

CHAPTER TWO

2. Experimental

2.1 Apparatus

2.1.1 Flow Injection Analyzer

The Alitea USA/FIA Lab (Medina, Washington, USA) apparatus was used for all FI-experimental work. The apparatus consists of the following components:

(1) Pump: A high quality peristaltic pump (C4V, Alitea, WA,USA) with cassette drive is used. It features eight stainless steel rollers on individual bearings with individual cassettes for as many as four pump tubings and a gearbox with a double bearing shaft. Pump tubings of 1.02 mm i.d. (Cole Parmer, Chicago, USA) were used.

(2) Injector: A Rheodyne model 5041 4-way poly(tetrafluoroethylene) (PTFE) rotary valve (Cotati, CA, USA) mounted on an angle bracket in the injector position of the FIA Lab apparatus was used to inject a definite volume of sample .

(3) Reactor module: Consisting of 0.5 mm i.d. PTFE tubing (Thermoplastic Scientific, NY, USA) of different length was used. The length of this reaction coil is chosen as appropriate and coiled in a way to enhance radial mixing.

(4) Detector: A Spectronic Mini-20 spectrophotometer (Milton Roy, Rochester, NY, USA), with a grating monochromator detector, a Unovic

ultra-micro-flow-through cell (Unovic instruments, NY, USA) of 20 µl size and with a pathlength of 1.0 mm.

(5) Recorder: Model 0555 single channel-strip-chart recorder (Cole Parmer, Chicago, USA) was used for peak absorbance-time recording.

2.1.2 Sequential Injection Analyzer

The sequential injection analyzer was constructed from the following components:

(1) Peristaltic pump (C4V, Alitea USA., Medina, WA, USA.).

(2) A Valco - 10 port selector valve (cheminert, Valco instruments, Houston, TX) is used to select the flows. Upchurch fittings (Upchurch, Oak Harbor, WA, USA) were used to lock unused ports.

(3) The holding coil and the reaction coil tubings as well as the tubings connecting the different units were made of PTFE (0.8 mm i.d.). Teflon nuts and ferrules (Upchurch, Oak Harbor, WA, USA) were used to fit these tubes into the different parts of the apparatus. Pump tubings were Phar Med™ 1.02 mm i.d. (Upchurch Oak Harbor, WA, USA) held on the pump rollers by FIA peristaltic pump tubing adapters (Upchurch, Oak Harbor, WA, USA).

(4) A Spectronic Mini-20 spectrophotometer (Milton Roy Co., Rochester, New York), equipped with a Unovic Ultra-micro-flow cell of 20 µl and 1.0 mm path-length is used.

(5) A personal computer (Austin computers system, Austin, TX, USA) working at 33 MHz, and equipped with a 120 Mb hard disk, 4 Mb RAM and

VGA Graphics, is used to monitor digitally the pump and the valve. The communication between the computer and the external devices was expanded by a general purpose I/O board (Model ADA-110, Real Time Devices (RTD), State Collage, PA, USA). The computer is also used to collect the data, alternatively the data is recorded by Model 0555 single channel strip-chart recorder (Cole Parmer, instrument Co., Chicago).

Perkin Elmer Lambda 5 UV/Visible Spectrophotometer equipped with 10.00 mm cells was used for preliminary investigations

2.2 Reagents

Cerium(IV)solution: Stock solution (0.10 M) was prepared by dissolving 16.0 g of cerium (IV) sulfate dihydrate (Merck, UK) in 250 ml of 0.05 M sulfuric acid. This solution is usually used after 24 hr.

Iron(III) Solution. A 0.10 M iron(III) solution was prepared in 0.05 M sulfuric acid by dissolving exactly about 24.9 g of dried ammonium ferric sulfate $NH_4 Fe(SO_4)_2.12H_2O$ (BDH, Poole, UK) in 0.05 M sulfuric acid in a 500 ml calibrated flask and used after 24 hours to guarantee complete dissolution.

Pd(II) Solution: A stock solution of 0.025 M was prepared by dissolving 0.4430 g of anhydrous palladium chloride (Fluka) in 50 ml hydrochloric acid solution of 0.04 M. The mixture was warmed at 80°C in a water bath

until all the palladium chloride solid is dissolved, then cooled to room temperature and made up to 100 ml with water.

Iron(II) solution: A stock solution of 0.2865 M was prepared by dissolving about 11.0g of ammonium ferrous sulfate $\{Fe(NH_4)_2(SO4)_2.6H_2O\}$ in 0.070 M HCl in 100 ml volumetric flask.

Ammonium sulfate: 0.20 M solution was prepared by dissolving about 13.4 g of AnalaR ammonium sulfate in water in 500 ml volumetric flask.

Hydrochloric acid: solution (0.1-2.0 M): Prepared by diluting AnalaR concentrated acid.

Sulfuric acid: (1.0-5.0 mol dm^{-3}). This was prepared by diluting AnalaR concentrated acid.

Pure drug solutions. The drugs included in this study together with the supplier name and other relevant information are given in table 2.1. Stock solutions of 1000-2000 ppm of pure analytic generic form of each of these drugs, were prepared by dissolving the drug in water or dilute acid solution at room temperature from which working solutions were prepared by appropriate dilution.

tablets formulations: The stock solutions were prepared by crushing five to ten tablets priorly weighed out, and dissolving an amount of the powder equivalent to a certain mass of the tested drug in water or dilute acid solution. The mixture was warmed for 15-30 minutes in a water bath, shaken for five minutes, filtered through an ordinary filter paper, washed with the hot water or hot acid solution several times and the filtrate plus

Table 2.1 List of drugs used and their suppliers.

Generic name	Supplier name	Batch number
Chlorpromazine	Rhone-Poulenc	E99
Promethazine-HCl	Rhone-Poulenc	W3021
Perphenazine	Rhone-Poulenc	D11
Trimeprazine	Rhone-Poulenc	CD14014
Ciprofloxacin	Bayer	09867
Norfloxacin	Merk, Sharp and Dohme	___
Bromazepam	Hofmann-La Roche	___
Procanamide-HCl	Aldrich	___
Oxprenolol	Ciba-Giegy	012482

washings were completed to the mark in a calibrated flask. Appropriate dilutions were made from these stock solutions.

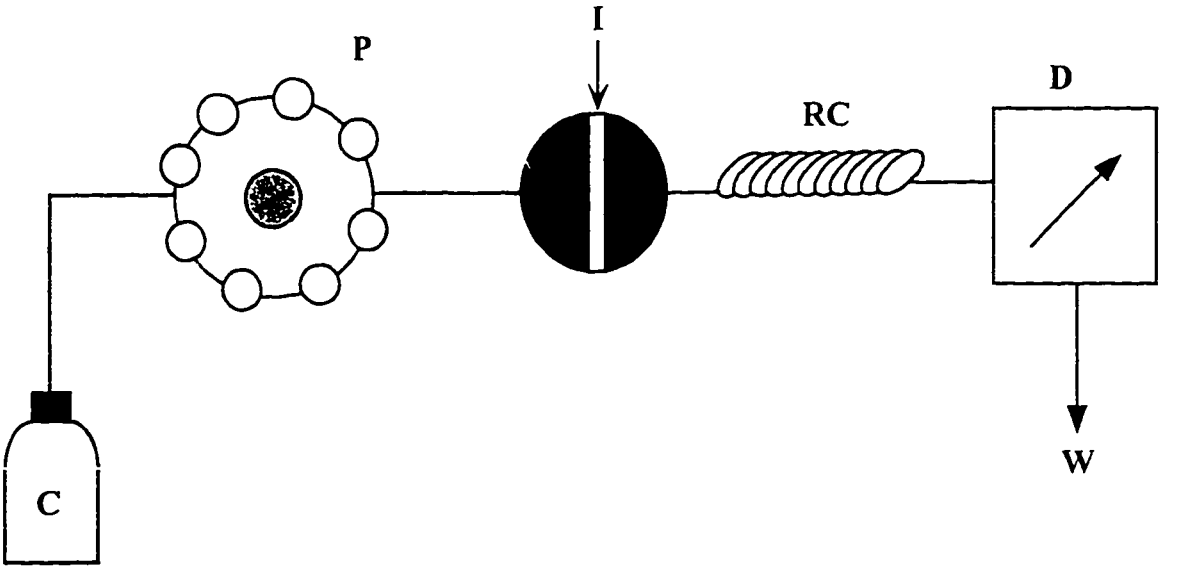
Syrup and Injections: Tested solutions were prepared by diluting a pipetted amount of the injection or the syrup in a calibrated flask with water or dilute acidic solution without any treatment. Working solutions are prepared from these solutions by further dilution.

2.3 Procedure

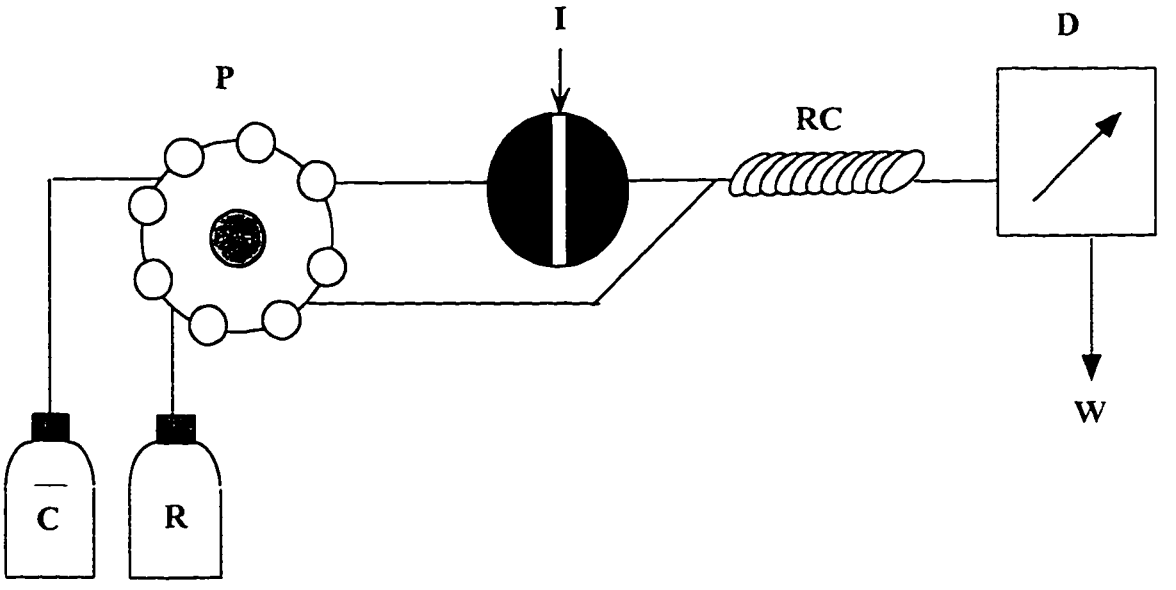
2.3.1 FIA-Procedure

Two types of FI-manifolds are used in this work, single-line manifold and double-line manifold. The single-line manifold (Figure 2.1) is the simplest FIA-system consisting of only one tube through which the premixed reagent carrier solution moves towards the flow-through-detector. In the double-line manifold (Figure 2.2) one confluence point is present past the injector valve where the second reagent is added and mixed with the carrier stream.

Using either manifold, the carrier solution was allowed to flow for few minutes at a certain flow rate, determined by the speed of the pump, absorbance was adjusted to zero in the spectrophotometer and the base-line of the chart recorder was allowed to stabilize. The sample was then injected into the flowing stream and the reaction was allowed to take place in the reaction coil. The developed colored compound was allowed to



Figuer 2. 1 Single line manifold: P, peristaltic pump; I, injector port; RC, reaction coil; D, flow-through detector; W, waste.



Figuer 2. 2 Double line manifold;C, carrier; R, reagent; P, peristaltic pump; I, injection port; RC, reaction coil; D, detector; W, waste.

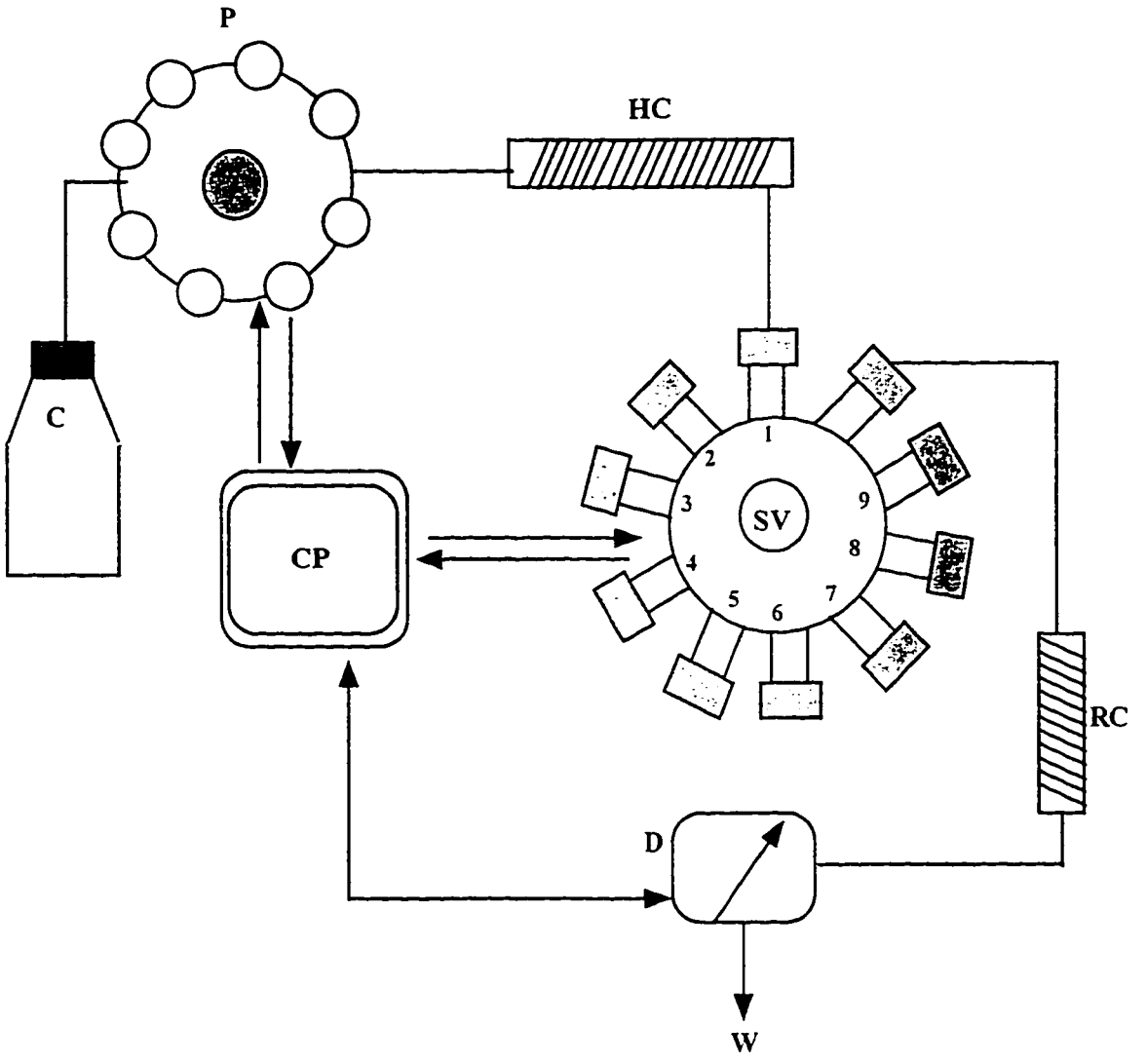
flow at the same flow rate of the carrier stream through the micro-flow through cell in the spectrophotometer at the selected wavelength.

2.3.2 SIA-Procedure

Figure 2.3 shows the SI-manifold used in this study, the components of which has been outlined above. The procedure starts by nesting reagents, samples and carrier(wash) solutions around the selector valve (SV). A certain amount of each of these solutions was first aspirated into the lines from ports by selecting each port at time. This step was aimed to fill these lines with appropriate solutions. The excess solution introduced into the holding coil (HC), one at a time, was expelled through port 9 to auxiliary waste. The steps of the analysis procedure using SIA are then fed into the computer as follows:

1. The carrier solution is pumped through port 1, by setting the pump in the forward direction for a 25-45 seconds in order to flush the system with the carrier solution.
2. A predetermined volume of the reagent solution was aspirated through port 2 into the holding coil (HC)
3. Drug solutions were aspirated into the holding coil via ports 3-8, one at a time. Both of the last two steps were achieved by setting the pump in the reverse mode.

Finally the composite zone is propelled by the carrier solution through port (1) to the reaction coil and then into the detector. The data was then acquired by the computer and transferred to a plotting software for further manipulations.



Figuer 2. 3 SIA-manifold; C, carrier; P, peristaltic pump; HC, holding coil; SV; selector valve; RC, reaction coil; D, detector, CP, computer; W, waste.

In each of the above steps the volume of the solutions aspirated were determined from the time of aspiration and the volumetric flow rate of the pump, which was 29.5 $\mu l\ s^{-1}$ in this study.

2.3.3 SIA-Kinetics

The SIA manifold as represented by Figure (2.3) with the pump operable at a flow rate of 29.5 $\mu l\ s^{-1}$, volumes from standard solutions could be withdrawn for certain time precisely adjustable by the pump stroke through the computer board and the following steps were performed :

1. Iron(II), hydrochloric acid and the drug were connected to the selector valve through ports 2, 3 and 4 respectively. Water is pumped as a carrier solution through valve 1 in the forward mode to waste to the detector for 25.0 s.

2. About 80 µl each of the above reagents were around the selector valve were introduced sequentially into the holding coil in the reverse mode for 3.0 s, then transferring the excess together with some carrier to auxilliary waste through valve 9 in the forward mode for 8.0 s.

3. In the analysis method, Iron(II) and hydrochloric acid concentrations are kept constant by transferring fixed volumes previously calculated from known concentrations of these reagents and aspirated into the holding coil in a reverse mode for 9.0 and 4.0 s respectively which are then

pushed into the mixing chamber together with the carrier through valve 8 for 25.0 s. This phase is referred to as the transfer volume (V_T).

4. A certain known volume of the test solution is withdrawn into the holding coil then pushed into the mixing chamber together with some carrier solution for 25.0 s in a separate step referred to as the sample volume (V_S). Note that both (V_t) + (V_S) were always pushed to the mixing chamber for 50.0 s to keep its volume constant through the experiment.

5. A known volume of the aliquot solution is withdrawn from the mixing chamber and transferred to the holding coil in a reverse mode for 7.0 s, then pushed in the forward direction for 12.0 s to the detector for signal monitoring. This phase is referred to as the analysis volume (V_A).

6. For kinetic measurements at variable times, reagents and drug solutions are arrested for the predetermined time in the mixing chamber prior to the analysis volume is withdrawn.

7. For determining reaction orders with respect to one reactant, all other components are kept constant and treated as V_T. The one varied is considered the (V_S) and the process is repeated by taking different volumes for (V_S) from a single standard solution so that the kinetics monitored corresponds to different required solutions.

2.3.4 Simplex Optimization

The factors assumed to influence the response in flow techniques were divided into two classes: physical (instrument) factors, and these include coil length, flow rate, and sample loop size, and chemical factors. The upper and lower bound of these factors will be discussed for each case separately in the coming chapter. The search algorithm employed in this work was the super-modified simplex technique owing to its speed and reliability and its easy application on PC-computers. The simplex procedure begins by feeding the boundary conditions to the computer, followed by the initial vertex. The initial vertex in each case was selected after a thorough investigation of the response surface. The computer then calculated the next vertex of the start simplex. The coordinates (factor settings) of the next vertex were adjusted and an experiment(s) was then run and the response was collected and fed to the program to calculate the next vertex. This procedure was repeated until the start simplex was obtained. Then the program continued calculating the coordinates of the reflected, contracted and expanded vertexes and in each case the conditions were adjusted and experiments were run and responses were collected. The procedure was halted when the termination criterion of the program was satisfied.

2.3.5 Experimental Design

The statistical experimental designs used in this work were:

1. 2^5 design, where 5 variables were investigated at two levels, these two levels will be referred to as high and low levels(see table 2.2).

2. 3^2 design, where two factors were investigated at three levels, high, center and low levels (see table 2.3).
3. A composite central design, where five factors were investigated at five different levels (see table 2.4).

In this chapter these designs will be displayed considering the coded levels only, where -1 denotes low level, +1 denotes high level and 0 denotes center (intermediate) level.

The combination of factors of each experiment from these designs was adjusted and the response were then collected. These experiments are randomized by a computer program and are not necessarily run as they appear in these tables. All of the experiments corresponding to each of these designs were run within the same day in order to minimize any variation due to operations at different days. The statistical analysis was then performed on the collected data utilizing statistical software.

2.4 Software Packages

1. COPS (Chemometrical optimization by simplex) program, Elsevier Scientific Co., The Netherlands
2. SAS(Statistical Analysis Systems) program, SAS institute, North Carolina, USA.
3. Statgraphics, Statistical Graphics Co. USA.
4. Sigmastat, Jandel Scientific,

Table 2.2 2^5 factorial design.

Exp. #	Factor A	Factor B	Factor C	Factor D	Factor E
1	-1	-1	-1	-1	-1
2	1	-1	-1	-1	-1
3	-1	1	-1	-1	-1
4	1	1	-1	-1	-1
5	-1	-1	1	-1	-1
6	1	-1	1	-1	-1
7	-1	1	1	-1	-1
8	1	1	1	-1	-1
9	-1	-1	-1	1	-1
10	1	-1	-1	1	-1
11	-1	1	-1	1	-1
12	1	1	-1	1	-1
13	-1	-1	1	1	-1
14	1	-1	1	1	-1
15	-1	1	1	1	-1
16	1	1	1	-1	-1
17	-1	-1	-1	-1	1
18	1	-1	-1	-1	1
19	-1	1	-1	-1	1
20	1	1	-1	-1	1
21	-1	-1	1	-1	1
22	1	-1	1	-1	1
23	-1	1	1	-1	1
24	1	1	1	1	1
25	-1	-1	-1	1	1
26	1	-1	-1	1	1
27	-1	1	-1	1	1
28	1	1	-1	1	1
29	-1	-1	1	1	1
30	1	-1	1	1	1
31	-1	1	1	1	1
32	1	1	1	1	1

Table 2.3 3^2 Factorial design.

Experiment No.	Factor A	Factor B
1	-1	-1
2	0	-1
3	1	-1
4	-1	0
5	0	0
6	1	0
7	-1	1
8	0	1
9	1	1

Table 2.4 Central composite design for five factors

Exp.#	Block	Factor A	Factor B	Factor C	Factor D	Factor E
1	1	0	0	0	0	0
2	1	-1	-1	-1	-1	-1
3	1	1	-1	-1	-1	-1
4	1	-1	1	-1	-1	-1
5	1	1	1	-1	-1	-1
6	1	-1	-1	1	-1	-1
7	1	1	-1	1	-1	-1
8	1	-1	1	1	-1	-1
9	1	1	1	1	-1	-1
10	1	-1	-1	-1	1	-1
11	1	1	-1	-1	1	-1
12	1	-1	1	-1	1	-1
13	1	1	1	-1	1	-1
14	1	-1	-1	1	1	-1
15	1	1	-1	1	1	-1
16	1	-1	1	1	1	-1
17	1	1	1	1	1	-1
18	1	0	0	0	0	0
19	1	-1	-1	-1	-1	1
20	1	1	-1	-1	-1	1
21	1	-1	1	-1	-1	1
22	1	1	1	-1	-1	1
23	1	-1	-1	1	-1	1
24	1	1	-1	1	-1	1
25	1	-1	1	1	-1	1
26	1	1	1	1	-1	1
27	1	-1	-1	-1	1	1
28	1	1	-1	-1	1	1
29	1	-1	1	-1	1	1
30	1	1	1	-1	1	1
31	1	-1	-1	1	1	1
32	1	1	-1	1	1	1
33	1	-1	1	1	1	1
34	1	1	1	1	1	1
35	1	0	0	0	0	0
36	2	0	0	0	0	0
37	2	-2.4378	0	0	0	0
38	2	2.4378	0	0	0	0
39	2	0	-2.4378	0	0	0
40	2	0	2.4378	0	0	0
41	2	0	0	-2.4378	0	0
42	2	0	0	0	0	0
43	2	0	0	2.4367	0	0
44	2	0	0	0	-2.4378	0
45	2	0	0	0	2.4378	0
46	2	0	0	0	0	-2.4378
47	2	0	0	0	0	2.4378
48	2	0	0	0	0	0

Two other programs were written in C-language by us and employed for the calculation of stoichiometry and formation constants of complexation reactions. The Jobcon program,[49] was utilized to calculate stoichiometries and formation constants from continuous variation data. Merlet program,[50] was modified and used to fit molar ratio data. The pseudocode of these two programs is given in Appendixes A and B respectively.

CHAPTER THREE

3. Chemometric Optimization

3.1 Determination of Promethazine- HCl in Drug Formulations *

Promethazine-HCl is dimethyl-[1-methyl-2-(phenothiazine-1-yl)ethyl] amine-HCl. It is commonly prescribed for its antihistaminic action. Currently it is often used after minor operations as an emetic, analgesic, sedative and hypnotic drug. Various methods for its determination have been reported in the literature; mainly, spectrophotometric, chromatographic and electrochemical methods. These have been recently reviewed(6). However, no method has been reported on the determination of promethazine by flow injection technique except recently using the metavanadate method(6) which was found to be suitable for other phenothiazines. The metavandate method also suffers interferences from excipients when applied to determination of the drug in pharmaceutical preparations. Moreover, the instability of the oxidized form of the product required the use of a milder oxidant and a computerized simplex optimization procedure for obtaining generous and reasonable conditions. The official BP method(51) for the assay of promethazineHCl in Tables and syrups is carried out by tritrating the sample in hydrochloric

* Sultan S. M., Suliman F. O.,Anal. Sci.,1992,8, 841.

acid followed by reacting with palladium(II) reagent, and measuring the absorbance of the resulting solution at 472 nm.

The present work describes a flow injection spectrophotometric method for the determination of promethazineHCl. The method involves the use of cerium (IV) to oxidize promethazine in sulfuric acid media to yield a reddish colored product that shows maximum absorbance at 515 nm. Promethazine was oxidized with the vanadate(6) yielding the same product absorbing at the same wavelength and was believed to be the dication diradical. Preliminary investigations revealed that this product was more stable than when vanadate was used thus encouraging the use of cerium(IV) for better reproducible results.

3.1.1 Optimization of Parameters

The FI-manifold used here is the single line manifold described in Chapter 2 (figure 2.1) . The chemical reaction system employed in this work is found to be somewhat complicated with respect to the formation and stability of the oxidized form of the drug. The reaction was found to be acidic and time dependent. A univeriate approach was employed to optimize the sample loop size which revealed that this parameter has no great effect on absorbance and 110 µl was considered to be optimal. Keeping this parameter constant, reaction coil length, cerium(IV) concentration, sulfuric acid concentration and flow rate parameters were optimized by utilizing the super modified simplex procedure.(26)

Table 3.1 Simplex optimization of chemical and FI- parameters for the determination of promethazineHCl.

Exp#	Coil length/cm	Flow rate/ ml min^{-1}	$[H_2SO_4]$ / M	[Ce(IV)]/ x 10^{-4}M	Peak absorbance
(1)	45	4.100	0.100	0.357	0.168
(2)	87	4.231	0.264	2.31	0.360
(3)	55	4.231	0.264	8.50	0.462
(4)	55	4.231	0.794	2.31	0.384
(5)	55	4.655	0.264	2.31	0.384
(6)	81	4.574	0.694	7.35	0.312
(7)	68	4.408	0.485	4.93	0.401
(8)	72	4.306	0.358	3.43	0.447
(9)	67	4.334	0.393	3.84	0.465
(10)	64	4.507	0.100	6.42	0.453
(11)	63	4.460	0.219	5.71	0.462
(12)	71	4.100	0.406	8.79	0.378
(13)	63	4.250	0.511	5.35	0.504
(14)	62	4.146	0.657	5.17	0.507
(15)	74	4.437	0.662	0.728	0.180
(16)	60	4.289	0.376	6.40	0.534
(17)	58	4.311	0.525	6.79	0.537
(18)	65	4.100	0.681	6.40	0.534
(19)	66	4.100	0.720	6.58	0.498
(20)	58	4.100	0.589	6.58	0.504
(21)	60	4.166	0.567	6.23	0.534

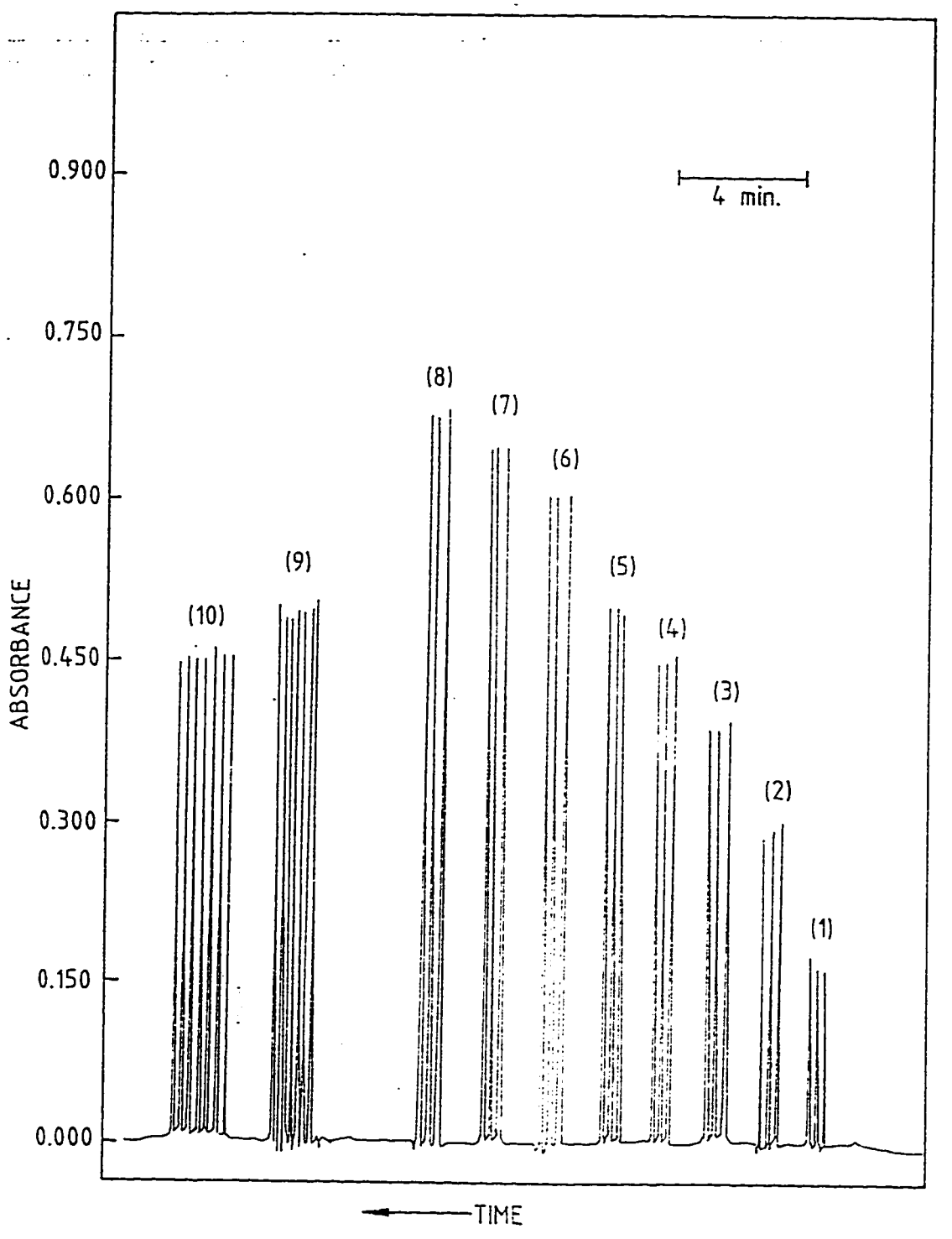


Figure 3. 1 Typical FI plots, run in triplicate of promethazine solutions of (1) 60; (2) 80; (3) 100; (4) 120; (5) 140; (6) 160; (7) 180; (8) 200 ppm; (9) seven runs of 140 ppm Phenergan Tablets and (10) seven runs of 120 ppm Phenergan syrup.

Sensitivity, precision and sample throughput were considered in optimizing promethazineHCl determination.

The boundary conditions of lower and upper for each of the above four parameters optimized being $1.0x10^{-5}$ and $2.5x10^{-3}$ M cerium(IV), 0.10 and 2.5 M sulfuric acid, 45 and 200 cm coil length and 4.1 and 6.0 ml/min. flow rate, were defined according to preliminary investigation of the kinetics of the reaction and fed into the computer. The first point conditions and response were fed and the program automatically calculated vertexes of the starting simplex: the first five experiments are as in Table (3.1). The optimization was halted after 21 experiments and the average of the top three responses and the corresponding parameter values were calculated to obtain optimum conditions. These were found to be very close to maximum response values as follows: 62 cm coil length, $6.19x10^{-4}$ M cerium (IV) , 0.512 M H_2SO_4 and 4.25 ml min^{-1} flow rate.

From Table (3.1) and as the simplex progressed, several trends were observed for the four variables. The flow rate has no greater effect on response and this was confirmed by the reaction coil length which did not exceed the double initial length indicating that longer coil length leads to dispersion or disproportionation of the product at longer residence time. Generally a flow rate of 4.2 ml min^{-1} is regarded as optimal in flow injection; lower than this rate the sample throughput starts to decrease significantly. The response increased with cerium(IV) concentration and reached maxima at $6.19x1^{-4}$ M; beyond which reaction rate increases significantly leading to quick disproportionation of the product and lower

absorbance was attained. The increase in sulfuric acid concentrations decreases reaction rate resulting in the stability of the product especially at 0.5 M.

3.1.2 Calibration Graph

Various standard solutions were run in triplicates with a typical example represented in figure 3.1. Peak width at baseline was calculated to be 18 seconds thus defining the sampling frequency as 200 samples per hour. The narrow peak width at 60% peak height indicates minimal dispersion. A relative standard deviation of 0.80% was obtained for repeated different

fixed concentrations of promethazineHCl showing good reproducibility of the results. Linear regression analysis of peak absorbance versus promethazine concentrations gave a very small intercept, a good correlation coefficient of 0.99 and a slope of the following equation: $A = -0.03636 + 0.003876 \times 10^{-3} C$. The calibration graph is linear over a wide range of promethazineHCl concentrations of 60-200 ppm.

3.1.3 Applicability and Interferences

This method is applied to the determination of promethazineHCl in the proprietary drugs, Phenergan Tablets and Phenergan syrup (peak 9 and 10, respectively in figure 3.1) ; both formulations contain starch and glucose as excipients. The same batches were analyzed by the BP method(51) and the results were statistically compared by calculating percent recoveries,

Table 3.2 A statistical comparison of the results of determination of proprietary drugs containing promethazineHCl by the present method compared with those obtained by the official BP method(51).

Proprietary drug	Active material	Recovery ± SD % *		t **
		FIA	BP	
Phenergan Tablets, (Specia, France)	Promethazine-HCl, 25 mg.	99.7 ± 0.81	100.1 ± 0.34	1.4
Phenergan syrup (Specia, France)	Promethazine-HCl, 1000 ppm	102.2 ± 0.73	101.9 ± 0.94	0.93

* standard deviation calculated as a mean for 5 determinations.

** student t-test calculated, theoretical value = 2. 78 (P= 0.05)

standard deviations and the student t-test values as in Table 3.2. The present method showed the same degree of accuracy of the BP method. Results also show that the effect of the common drug fillers do not interfere with the determination.

The present method has the advantage of selectivity and simplicity over the BP and previous methods. The utilization of the super modified simplex procedure improved reagents consumption and increased sensitivity enabling the application in a wider range of promethazineHCl concentration. Moreover, the use of cerium(IV) reagent was applied without complication for the oxidation of the drug.

3.2 Determination of Ciprofloxacin Antibiotic in Drug Formulations*

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1 piperzinyl-3-quinolone carboxylic acid] is the reference substance for the modern fluoroquinolones that marks the beginning of a new era in antibacterial chemotherapy(21). It is the cyclopropyl residue at position 1 of the quinolone system that gives a considerable improvement in antibacterial activity compared with the corresponding fluoroquinolone of the ethyl residue at the same position(22). It is chemically obtained *via* the combined acylation and arylation (aracyclation) of enamines and enhydrazines with *o*-halo-(het) aroyl halides(52, 53). Its mode of action is thought to be through blocking bacterial DNA replication and transcription

* Sultan S. M., Suliman F. O., Analyst, 1992, 117, 1523.

by inhibiting DNA gyrase, ultimately giving rise to cell lysis. Because of this special mechanism of action, it is considered the most active broad spectrum antibiotic effective against Gram-positive-Gram negative pathogens to combat the infections caused by microorganisms that are resistant or multiresistant to other antimicrobials, such as aminoglycosides, tetracylcines or β-lactams. It was marketed the first time in 1987 and registered in the Kingdom recently.

Methods for the determination of ciprofloxacin in pharmaceutical preparations are not yet available in the literature nor in the British or United States Pharmacopoeia. However, chromatographic methods for its assay in biological samples in serum and urine are now numerous[54-60].

A flow injection spectrophotometric method for the determination of ciprofloxacin using iron(III) as a complexing agent in sulfuric acid media will be described.

3.2.1 Reaction Kinetics and Mechanism

Trials were successfully carried out to explore a simple chemical system by the versatile FI-micro technique that works smoothly for the determination of such an important new drug. A recent batch method based on the reaction of iron(III) with tetracycline [8,61] antibiotics and the clinical and chemical interaction between iron preparations with ciprofloxacin were recently investigated by Kara et al[62] led to the development of the reaction of iron(III) with ciprofloxacin and the exploration of the present method. Other metal ions such as iron(II), copper(II), calcium(II) and aluminum(III) were investigated but all gave

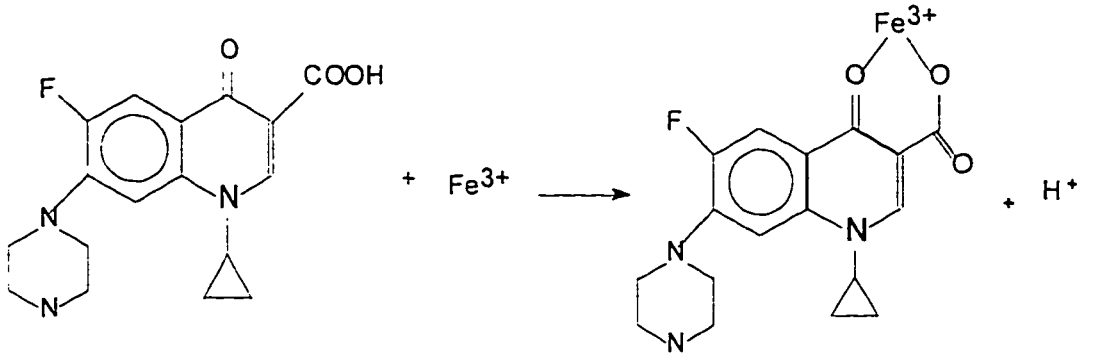
negative results. Iron(III) was found to react instantaneously to form a highly stable colored complex having an extinction coefficient of 1256.9 L mol^{-1} cm^{-1} in 0.023 M H_2SO_4 at maximum absorbance at 447 nm and remains unchanged for more than one week.

It was observed that the reaction is fast at room temperature and independent of sulfuric acid concentration. However, it was also observed that the intensity of the colored complex increased with a decrease in the concentration of the acid, indicating dissociation of the complex at higher acidities.

3.2.2 Stoichiometry

The Job's method [45] of continuous variation was employed under the working conditions of 0.023 M sulfuric acid concentration and at room temperature to establish the reaction stoichiometry. It is evident from the results obtained and the typical plot in figure 3.2 that the ratio of ciprofloxacin to iron(III) was always 1: 1.

Although the 1: 3 iron(III) : ligand complexes are usual, but the Job's plot in figure 3.2 indicates 1 : 1 complex. However, there was steep rise in absorbance when lower drug concentrations were used while a gradual decrease in absorbance was observed when high drug concentrations were used. This possibly suggests higher complex formations: although this is not strongly evident it indicates the possibility of existence of higher complexes as well as 1 : 1 complex which is dominant and forms the basis of this method.



Scheme 3.1 Reaction of ciprofloxacin with iron(III)

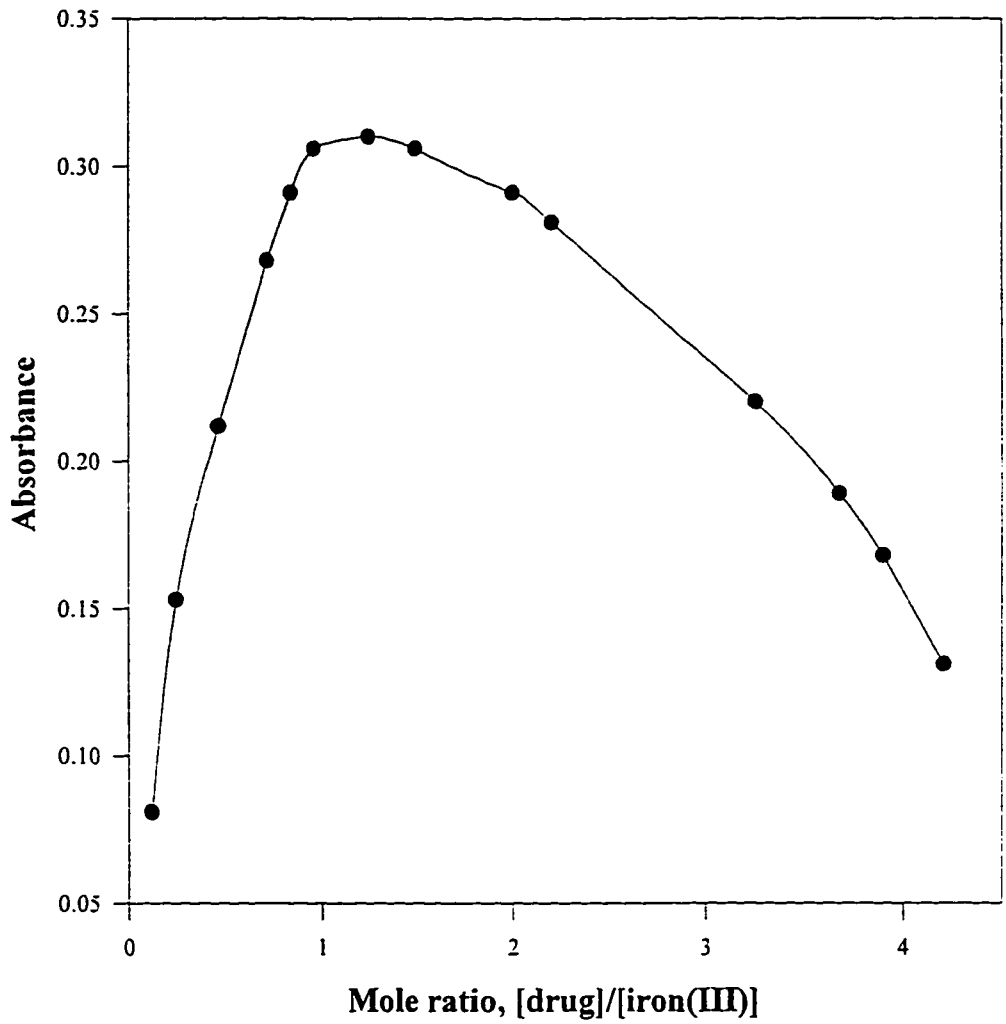


Figure 3.2 Job's plot of [Iron(III)] = [Ciprofloxacin] = 1.23 x 10^{-3} M; the maximum volume of each solution was 5 ml; 25 ml calibrated flask was used and each time it is filled to the mark with 0.023 M sulfuric acid.

it is suggested that the complexation is a fast step reaction in which iron(III) directly enters the active carboxylic acid and the adjacent keto group in the 3,4 positions respectively thus forming a six membered ring according to scheme 3.1

The mechanism offered here is in agreement with the observation that the complex dissociates at higher acidity as a result of the reaction favoring the backward direction. The confirmation of this reaction path would necessitate extensive pharmacokinetic studies to establish the effect of iron(III) chelation on the antibacterial activity of ciprofloxacin *in vivo*.

3.2.3 Simplex Optimization

The super modified simplex procedure (26) was successfully utilized for the optimization of four parameters: reaction coil length, flow rate, sulfuric acid concentration and iron(III) concentration, keeping the sample loop size constant at 110 μl throughout since it has no great effect on the results. The program starts by feeding the computer with the boundary conditions for each of the above parameters such as 45-400 cm for coil length; 4.1-6.00 ml min^{-1} flow rate; 0.005-0.50M sulfuric acid and 0.0001-0.040 M iron(III). The program was then supplied by the initial experimental conditions as No.1 in Table (3.3) to generate the initial simplex of (n+1) vertices i.e. 5 experiments. The response function was

chosen to measure the system performance by which sample throughput and sensitivity were all considered in one expression as follows:

$$R = \frac{A_{exp}}{A_{base} \cdot t_b} \qquad 3.1$$

Where R is the response function, A_{exp} absorbance at the peak maximum for experimental initial conditions, A_{base} is absorbance of the baseline reference conditions, t_b is the peak width at base line.

The response function is related to sensitivity by the term (A_{exp}/A_{base}), and to sample throughput by the value of t_b. A total of 38 experiments were generated to reach optimum conditions by injecting 110 µl of 400 ppm of the drug standard solution. Figure 3.3 shows a scatter diagram of the progress of the simplex. It is quite clear that there was a gradual improvement in the response function (by a factor of 10 from initial simplex) indicating a successful application of the program to the system optimization.

Optimum conditions were obtained by calculating the average of six parameters giving rise to the top six responses as introduced in Table (3.3) following the five initial simplex experimental conditions.

To justify taking average of conditions of the six experiments with top response functions, the following observations were considered: First, the maximum response functions obtained are almost identical and range between 2.448 to 2.586. Second, the highest response function among these experiments was obtained when the minimum acid concentration was used and this was a significant change in the acid concentration in comparison

Table 3.3 Initial and optimum conditions obtained by the simplex program for ciprofloxacin determination.

Exp#	Coil length / cm	Flow rate / ml min-1	$[H_2SO_4]$/ M	[Iron(III)] / mM	Response function
(1)	100	4.85	0.100	1.000	0.357
(2)	194	4.97	0.114	2.093	0.568
(3)	122	5.35	0.114	2.093	0.697
(4)	122	4.97	0.114	5.628	0.250
(5)	122	4.97	0.157	2.093	0.645
(30)	85	5.91	0.050	26.574	2.448
(33)	53	5.65	0.043	31.030	2.500
(34)	45	5.63	0.024	35.905	2.506
(35)	62	5.72	0.005	29.983	2.586
(36)	89	5.92	0.011	33.029	2.570
(37)	95	5.95	0.005	34.187	2.576

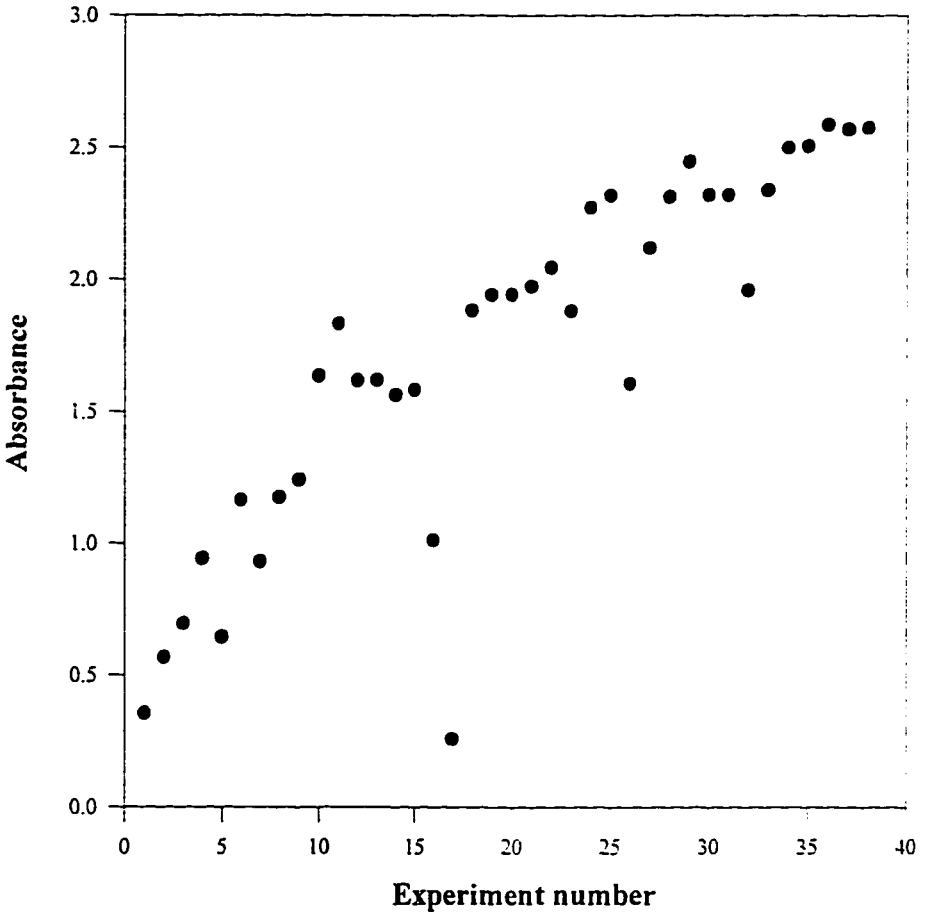


Figure 3.3 Progress of the response function of the simplex.

with that resulting in a small decrease of response function. Moreover, the change in the concentration of iron(III) was minimal at the level of the six experiments. Therefore, if we compare the response function values obtained by the initial simplex with those obtained by the last six experiments great effect of the reagent on response function can be seen reaching its maximum concentration and remaining almost constant at the best response function. Finally, averaging of conditions of the last six experiments was found to be the best compromise between longer coil length i.e., high dispersion, and higher flow rate i.e., high consumption of reagents, with little decrease, if any, in the response function.

It is concluded that the optimum conditions for ciprofloxacin determination are sample loop size 110 µl, coil length 72 cm, flow rate 5.80 ml min^{-1}, 0.023 M sulfuric acid and 0.00318 M iron(III).

3.2.4 Calibration Plot

Figure 3.4 was generated by running series of standard solutions of ciprofloxacin in triplicate from which absorbance versus concentration was found to be linear over a wide range of Ciprofloxcin concentration; that is between 50 to 500 ppm, with the following calibration equation:

$$A = 0.0116 + 0.001130\ C$$

Where A is absorbance, and C is concentration in ppm, with a correlation coefficient (r) of 0.99.

A weighted regression line was also plotted by giving minimum weighting ranging down to 0.1 for the lower and the upper concentrations with equal full weighting for the others in one trial, and also by giving full weighting to the mid point and decreasing by 0.1 in opposite directions in another trial, but all gave the same calibration equation as given above, thus confirming the excellent correlation of the points of the straight line obtained.

From these plots, which were obtained under the above optimum conditions, the peak width at the baseline was measured to be 14.5 s, thus defining the sampling frequency to be 250 samples per hour. Peak width was also measured at 60% peak height and found to be 2.4 seconds indicating minimal dispersion. Average relative standard deviation (r.s.d.) of 0.92% was obtained for repeated six determinations of 250 ppm indicating excellent reproducibility.

3.2.5 Application

The method was applied to the determination of ciprofloxacin in the proprietary drug: CiproBay Tablets and CiproBay infusion (Bayer) with seven runs presented by the groups of peaks 11 and 12 (Fig. 3.4) for both drugs respectively. Table (3.4) summarizes the results obtained with high percentage recoveries and a relative standard deviations similar to that obtained for the generic standard sample. The results obtained suggest that the method is suitable for the determination of ciprofloxacin in

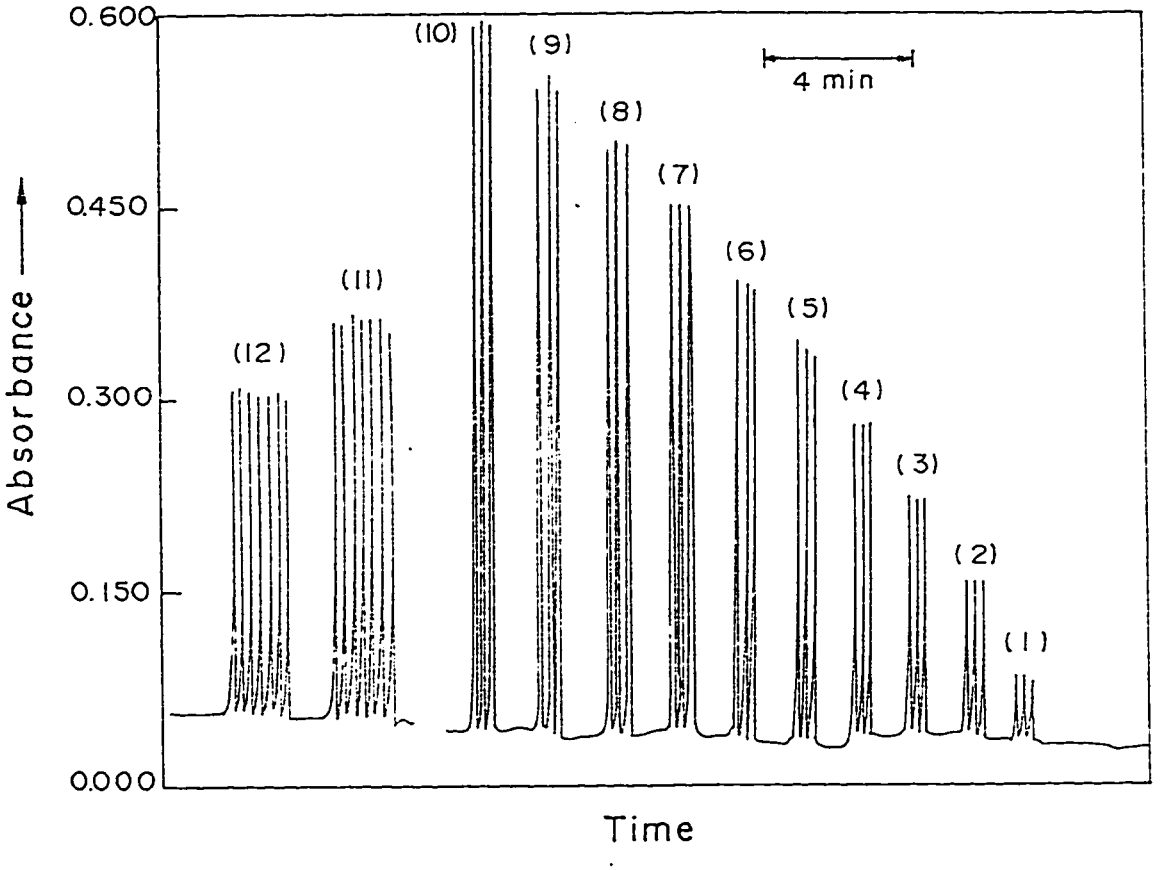


Figure 3. 4 Typical FI results in triplicate of ciprofloxacin standard solutions of; (1) 50; (2) 100; (3) 150; (4) 200; (5) 250; (6) 300; (6) 350; (7) 400; (8) 450; (9) 500; (10) seven runs of 255 ppm CibroBay Tablets; (11) seven runs of 206 ppm CibroBay infusion.

Table 3.4 Results of analysis of ciptofloxacin in proprietary drugs.

Proprietary dug	**Supplier**	**Active material**	**Taken/ ppm**	**Found/ ppm**	**recovery (%)**	**RSD * (%)**
CiproBay Tablets	Bayer	250 mg	255.0	259.4	101.8	0.96
CiproBay infusion	Bayer	2000 ppm	206.0	206.6	100.3	0.68

N.B. All calculations are based on seven determinations.

* for percent recovery.

pharmaceutical preparations without fear of interferences caused by excipients expected to be present in such formulations.

3.3 Determination of Norfloxacin in Drug Formulations*

Norfloxacin is chemically known as [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperzinyl)-3-quinolone carboxylic acid, has a broad spectrum antibacterial activity against Gram-positive and Gram-negative aerobic pathogens considered to be the first commercially available member of the modern fluoroquinolones(21, 22). Norfloxacin is specially prescribed for the treatment of complicated urinary tract infections. Its mode of action has advantages over the other common antibiotics. It is thought to inhibit the DNA gyrase enzyme that catalyses chromosomal DNA supercoiling as well as promotion of double stranded DNA breakage. The fluorine atom at the 6 position provides increased potency against Gram negative organisms and the piperazine moiety at the seven position is responsible for antipseudomonal activity.

Most assay methods for norfloxacin available in the literature are suitable for its determination in biological fluids(63-67). No official method has been reported for the determination of norfloxacin in pharmaceutical products. In this section a flow injection (FI) method for the assay of norfloxacin in drug formulations involving the use of iron(III) as a complexing agent in sulfuric acid media will be described. A single line manifold will be used with a spectrophotometer as a detector.

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* Sultan S. M., Suliman F. O., analyst, 1993, 118, 573.

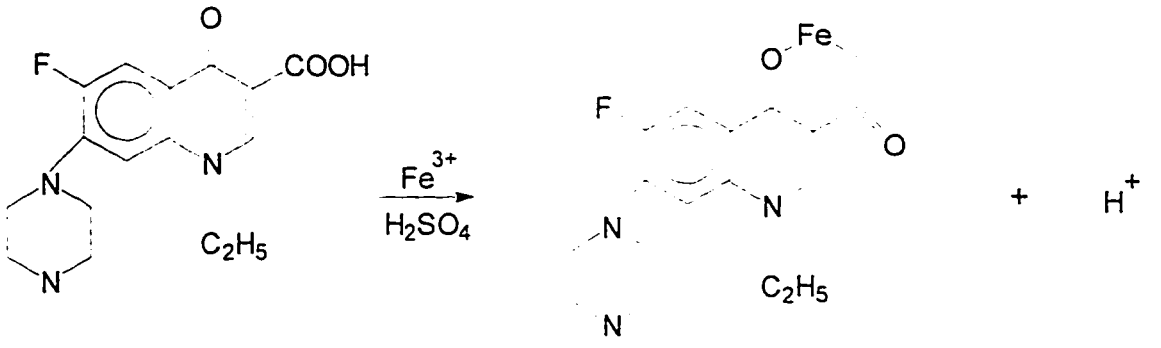
3.3.1 The Chemical System

The proposed FI method was based on the complexation reaction of the drug with iron(III) in sulfuric acid media. The complex thus obtained was found to be formed instantaneously, brown-red in color, absorbing at maximum at 430 nm and stable for more than two days.

The mechanism of the complex formation could possibly be similar to the one suggested earlier for the reaction of iron(III) with ciprofloxacin; a drug of the same fluoroquinolone type. Iron(III) enters the active carboxylic acid and the adjacent keto group in the 3,4 positions, respectively, thus eliminating a proton and forming a stable six membered ring as in scheme (3.2).

3.3.2 Optimization

As with any FIA technique, the effects of system and chemical parameters were studied. The factorial design[28,29] was utilized in order to establish the interaction between factors or parameters. By the effect of a factor we mean the change in peak height produced by a change in the level of the factor and by interaction between factors, we mean that the difference in response between the levels of one factor is not the same at all levels of the other factors. The statistic for testing any main effect or interaction is always formed by dividing the mean square for the main effect or interaction by the mean square error. That is:



Scheme 3.2 Reaction of norfloxacin with iron(III)

$$F = \frac{MS_{effect}}{MS_{residual}} \qquad 3.2$$

where MS is the mean square and equals SS/D.F where SS is the sum of squares i.e., SS = [sum of Response (high level) - sum of Response (low level)]^2 /32. D.F. is degrees of freedom. Different levels of significance (LS) were also indicated in the Table as 99.9% and 99.5%.

The five factors presumed to influence the peak height response such as sulfuric acid concentration, iron(III) concentration, sample size, flow rate and coil length were involved in the factorial design experiment. The fixed model analysis of variance was applied by designing a total of 2^5 i.e. 32 experiments where the five indicates the number of parameters and two represents the lower and higher levels of these parameters. These levels introduced in Table(3.5) were set on basis of preliminary investigation on the complexation reaction.

From the analysis of variance data in Table(3.6), and from the computed values of the main effect (F), it is clear that all parameters except the coil length parameter show significant interaction. The coil length was then fixed in all subsequent experiments to 45 cm long, which is considered a reasonable length. The other four parameters were investigated for optimization considering peak height alone, since sampling frequency and precision are reasonably accepted for quality assurance monitoring, as revealed by preliminary investigations carried out over a wide range of these parameters. The super modified simplex program(26) was employed as a search technique for the optimization.

Table 3.5 High and low levels of factors examined by the Factorial Design

Factor	Low level	High level
$[H_2SO_4]$ (A)	0.001M	0.500M
[iron(III)](B)	1.00 mM	30.00 mM
flow rate (C)	4.10ml/min	6.00ml/min
coil length (D)	45 cm	200 cm
sample volume (E)	110 µl	260 µl

Table 3.6 Analysis of variance

Source of variation	DF[a]	SS[b]	MS[c]	F[d]	LS[e]
$[H_2SO_4]$, A	1	9112.5	9112.5	380.18	99.9%
[iron(III)], B	1	8385.13	8385.13	349.84	99.9%
Flow rate, C	1	820.13	820.13	34.22	99.9%
Coil length, D	1	2.00	2.00	0.08	
Sample volume, E	1	450.00	450.00	18.7	99.5%
AB	1	5050.13	5050.13	210.70	99.9%
AC	1	120.13	120.13	5.01	99.5%
AD	1	112.50	112.50	4.69	99.5
AE	1	200.00	200.00	8.34	99.5
BC	1	4.50	4.50	0.19	
BD	1	10.13	10.13	0.42	
BE	1	55.13	55.13	2.30	
CD	1	36.13	36.13	1.51	
CE	1	45.13	45.13	1.88	
DE	1	40.50	40.50	1.69	
Residual[f]	16	383.5	23.97		
Total	31	25011.20			

[a] Degree of freedom

[b] sum of squares (see text)

[c] mean square = SS/DF

[d] $MS_{effect}/MS_{residual}$ (see text)

[e] level of significance

[f] Residual contains higher order interaction .

The results of the progress of the simplex as given in figure 3.5 and Table(3.7) show a gradual improvement in peak height and a clear leveling towards maximization. The results obtained by the simplex in this Table was obtained by injecting 270 ppm of the drug. The results indicates that peak height increases as sulfuric acid decreases, while iron(III), flow rate and loop size increase, reaching maxima when the values of these parameters were 0.043 M, 0.0271 M, 5.33 ml min^{-1} and 210 µl respectively, as shown by experiment number 21. Beyond these levels in the same direction, instability of the complex and hence lower absorbance value occur. Hence these values are considered to be the critical optimum conditions. Figure 3.6 is a scatter diagram, plotted from the simplex data, showing the effect of sulfuric acid concentration on the peak absorbance. The decrease of peak height at lower acid concentrations could be attributed to the competition of the hydroxyl ions favoring the reverse direction of the reaction, which could be regarded as a dominant effect over the increase of the other three parameters.

3.3.3 Analytical Appraisals

Series of standard solutions containing norfloxacin in the range 50 - 450 ppm were injected in triplicate with a typical run as in figure 3.7. The shift in baseline between injection sequence (1) and injection sequence (10) is a common problem in FIA measurements and could be avoided by calibrating to baseline intermittently so that routine quality assurance testing of drugs would not be affected. Peak heights with an average relative standard derivation of 0.57% for five repeated injections indicate

Table 3.7 Simplex optimization of chemical and FIA variables

Exp. #	[iron(III)]/ mM	Flow rate/ ml min^{-1}	$[H_2SO_4]$/ M	Sample volume µl	Absorbance
(1)	2.00	4.50	0.200	110	0.138
(2)	5.81	4.75	0.477	130	0.192
(3)	18.10	4.75	0.265	130	0.360
(4)	5.81	5.56	0.265	130	0.216
(5)	5.81	4.75	0.265	193	0.282
(6)	15.78	5.40	0.437	181	0.322
(7)	14.87	5.34	0.421	176	0.306
(8)	17.00	5.48	0.139	187	0.420
(9)	21.50	5.78	0.001	210	0.456
(10)	24.80	4.78	0.219	228	0.420
(11)	25.00	4.76	0.218	230	0.432
(12)	30.00	5.46	0.206	185	0.420
(13)	28.90	5.05	0.0010	194	0.464
(14)	22.29	5.00	0.186	167	0.408
(15)	29.81	5.47	0.0420	236	0.498
(16)	28.20	5.36	0.136	201	0.456
(17)	27.50	5.32	0.108	208	0.468
(18)	26.10	5.08	0.128	221	0.462
(19)	26.50	5.20	0.0920	217	0.480
(20)	23.70	5.84	0.120	242	0.464
(21)	27.10	5.33	0.0430	210	0.504
(22	24.60	5.56	0.0360	214	0.474

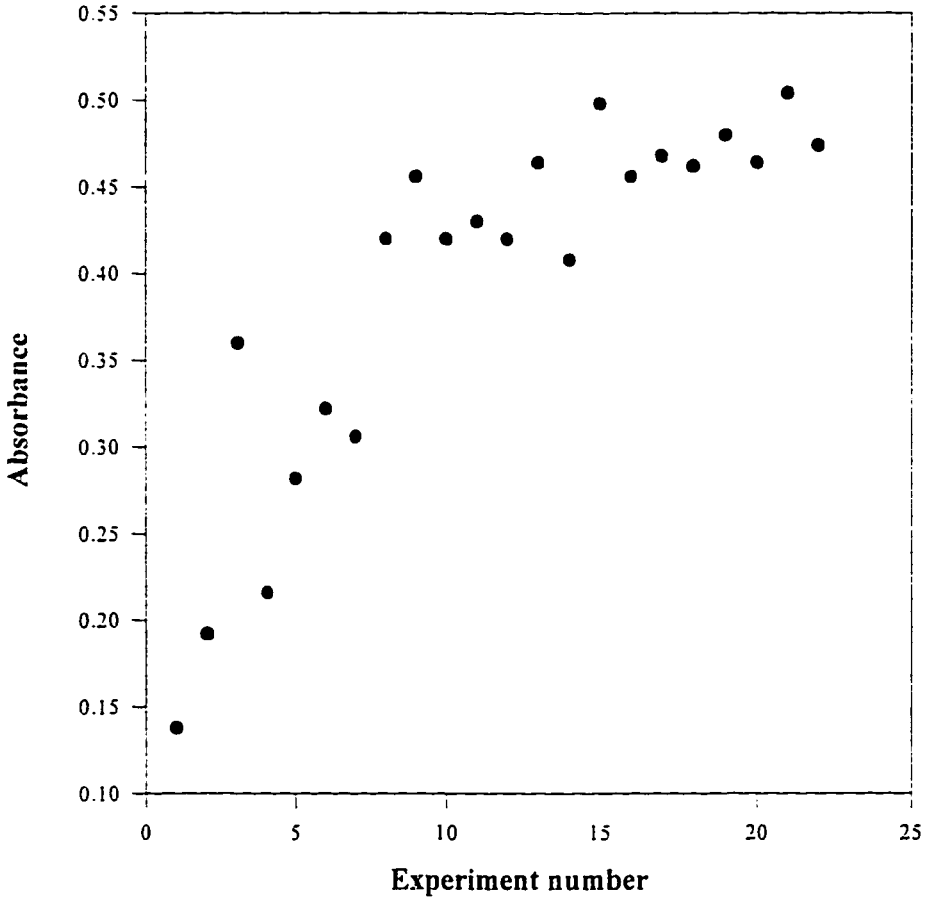


Figure 3.5 Response function progress of the simplex for norfloxacin.

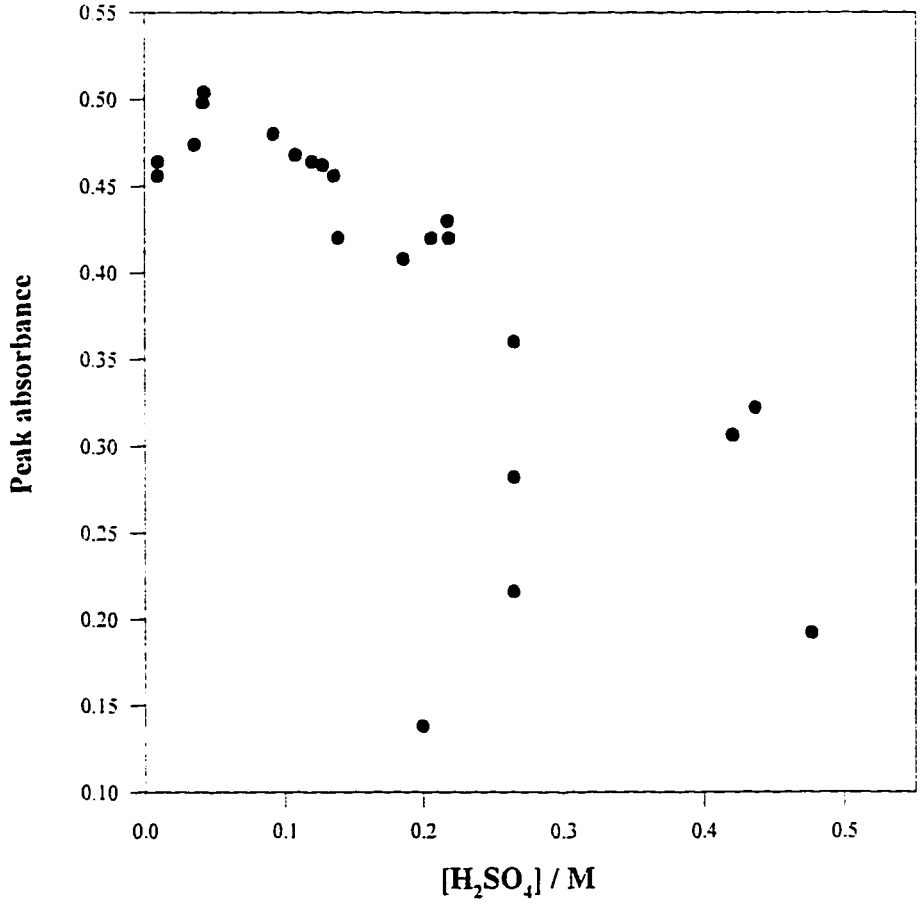


Figure 3.6 Effect of sulfuric acid concentration on peak absorbance.

very high reproducibility. The graph of absorbance (A) *versus* norfloxacin concentration (C ppm) was linear over the concentration range 50-450 ppm and a weighted regression line was plotted and that resulted in the following calibration equation : A = 0.0185 + 0.00168 C, with a correlation coefficient of 0.999.

A sample throughput of 140 h^{-1} could be attained; which was calculated by measuring peak width, which was found to be 26.4 seconds at base line, and 4.0 seconds at 60% peak height indicating minimal dispersion.

By using the conditions stated above the determination of norfloxacin in Noroxin Tablets was successfully employed by the five runs generated in peak number 10 in figure 3.7. The recovery of 100.7% and a relative standard deviation of 0.55% indicates that the method is suitable for the determination of norfloxacin in drug formulations without interferences from excipients such as starch and glucose.

The present method satisfies the literature need for a simple fast and sensitive procedure for the assay of norfloxacin in dug formulations suitable for routine quality control purpose. The method demonstrates typical and efficient application of the factorial design for the study of the effect of five factors as well as utilization of the supper modified simplex program for optimization of the interacting variables.

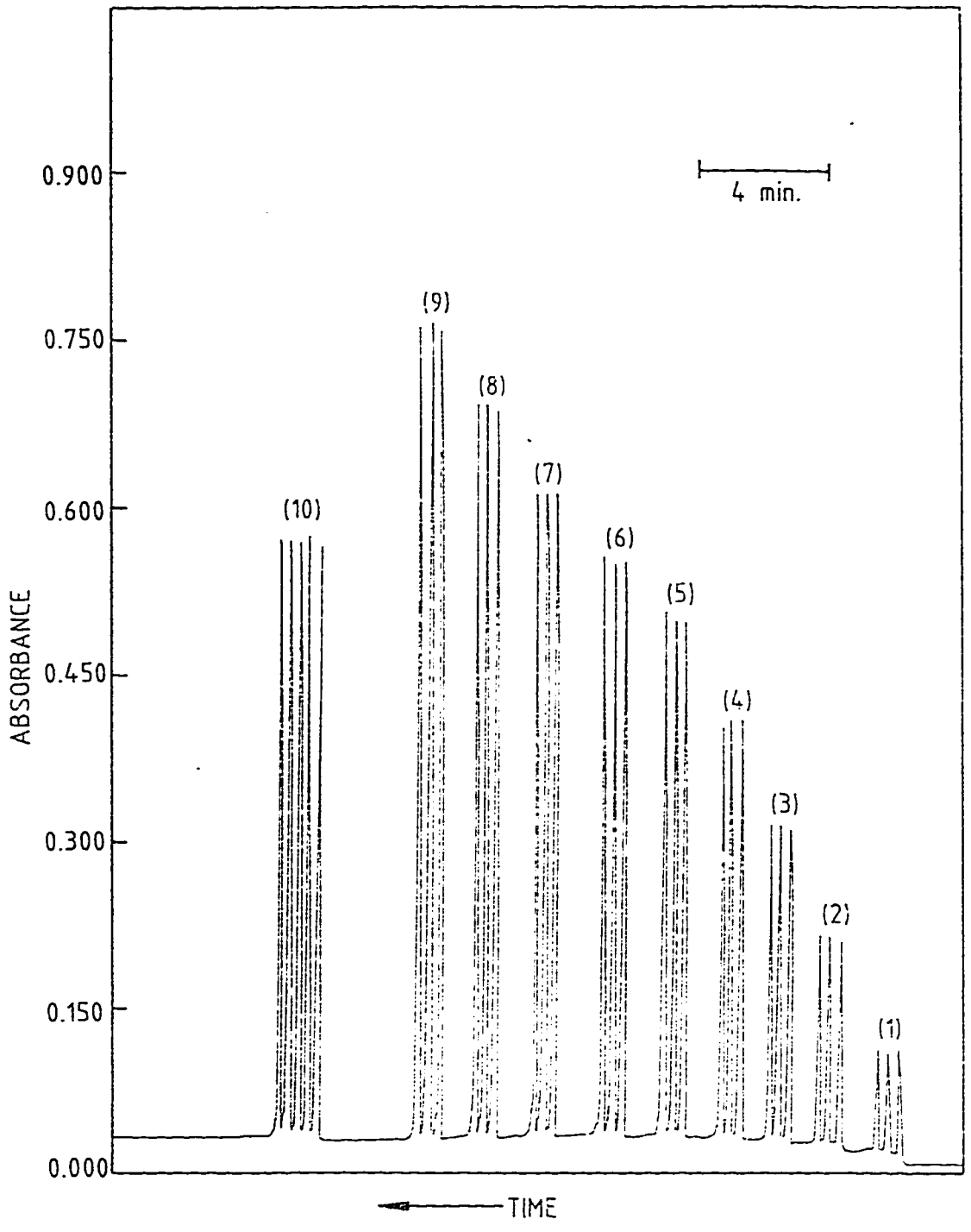


Figure 3.7 Recorder tracing for FI results of a series of standard norfloxacin solutions of (1) 50; (2) 100; (3) 150; (4) 200; (5) 250; (6) 300; (7) 350; (8) 400; (9) 450; (10) 315 ppm norflxacin (Noroxin tablets).

3.4 Assay of Procainamide-HCl in Drug Formulations*

Procainamide-HCl is chemically known as 4-amino-N-[2-(diethylamino) ethyl] benzamide hydrochloride. It is an anti-arrhythmic agent that has an action similar to those of procaine(68). It has also a depressant action on the heart similar to that of quinidine and also has anticholinergic properties. It is used intravenously for blood pressure control.

Various techniques for the assay of procainamide-HCl have been employed, including colorimetry(69-72), polarography(73) and chromatography (74-76). In the British Pharmacopoeia (BP) monograph(77) both the Tablet and injection formulations are assayed by dissolving in water, acidifying with hydrochloric acid and titrating versus sodium nitrite using ferrocyphen solution as indicator.

A flow injection (FI) method for the assay of procainamide HCl is described here. Cerium(IV) oxidant was used and the oxidized colored form of the drug was monitored spectrophotometrically at 480 nm, and a single-line manifold is used.

3.4.1 Chemical System

Preliminary investigations on the feasibility of oxidation of procainamide HCl with different oxidants revealed that cerium(IV) could

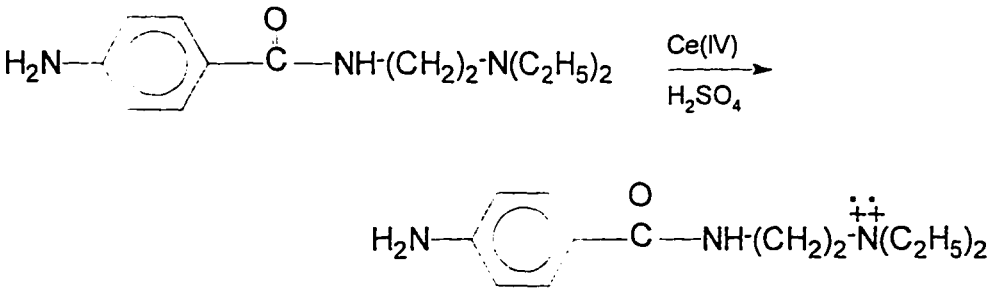
* Sultan S. M., Suliman F. O., Talanta, 1993, 40,

oxidize this compound yielding an amber product absorbing at maximum wavelength at 480 nm. The formation of this product was found to be instantaneous and appreciably unstable at higher sulfuric acid concentrations. The oxidation process takes place through the active site of N-(2-diethyl ethanol amine) group forming the dication diradical to which the colored product was attributed, a mechanism similarly postulated for the oxidation of the other β-Blockers[78,79] and could be sketched as in scheme 3.3

3.4.2 Optimization

Five factors presumed to influence the peak height were investigated, these are: sulfuric acid concentration, cerium(IV) concentration, sample size, flow rate and coil length. A 2^5 factorial design[28] was used to examine the significance of these factors on the sensitivity i.e. peak height. The settings of low and high levels of the factors in this design were based on the preliminary investigation of the reaction kinetics and these are shown in Table (3.8). The analysis of variance were introduced in Table (3.9). The F-value which is the criteria for significance was calculated by equation (3.2).

It is obvious from Table (3.9) that the only significant factors were cerium(IV) concentration and coil length. It is also obvious that cerium(IV) and sulfuric acid concentrations were significantly interacting. It is well known that a significant interaction might mask the significance of main effects[28]. Although, knowledge of the interaction is frequently more informative than knowledge of the main effect, it is usually useful to



Scheme 3.3 Reaction of procainamide with cerium(IV)

Table 3.8 High and low levels of factors examined by the Factorial Design

Factor	Low level	High level
$[H_2SO_4]$ (A)	0.100 M	1.50 M
[cerium(IV)] (B)	1.00 mM	7.00 mM
coil length (C)	45 cm	200 cm
flow rate (D)	4.10 ml/min	6.00 ml/min
sample volume(E)	110 µl	210 µl

examine the levels of one factor with levels of the other factors fixed to draw conclusions about the main effect of a factor. The least square means for the effect of sulfuric acid were found to be 49.0 and 52.0 mm at 0.100M and 1.50M, indicating that the effect of sulfuric acid is only important at different levels of cerium(IV) concentration.

As a result of the factorial design experiment the sample loop size and flow rate parameters were ruled out and for the economy of the concentration of reagents these parameters were fixed at 110 µl and 4.95 ml min^{-1}, respectively, and employed as such for the super modified simplex method to reach optimum conditions using coil length, sulfuric acid and cerium(IV) concentration as the simplex factors. The progress of the simplex is shown in figure 3.8, indicating a gradual improvement in peak height. From the simplex results introduced in Table(3.10), sixteen experiments were generated and experiment No. 14 gave the highest peak height from which the optimum conditions were found to be 114 cm coil length, 0.386M sulfuric acid and 6.351×10^{-3} M cerium(IV) solution. Figure 3.9 is a pseudo-three dimensional plot for the effect of sulfuric acid and cerium(IV) concentrations on the peak absorbance. This plot was generated by scaling the levels of both factors, obtained from simplex data, to ± 1, and then griding and extrapolating the data using an inverse-power method. It is clear from figure 3.9 and from Table (3.10) that the peak height increases with an increase in cerium(IV) concentration reaching a plateau region where it remains virtually constant. On the other hand, higher acid levels lead to a slight decrease in peak absorbance attributed

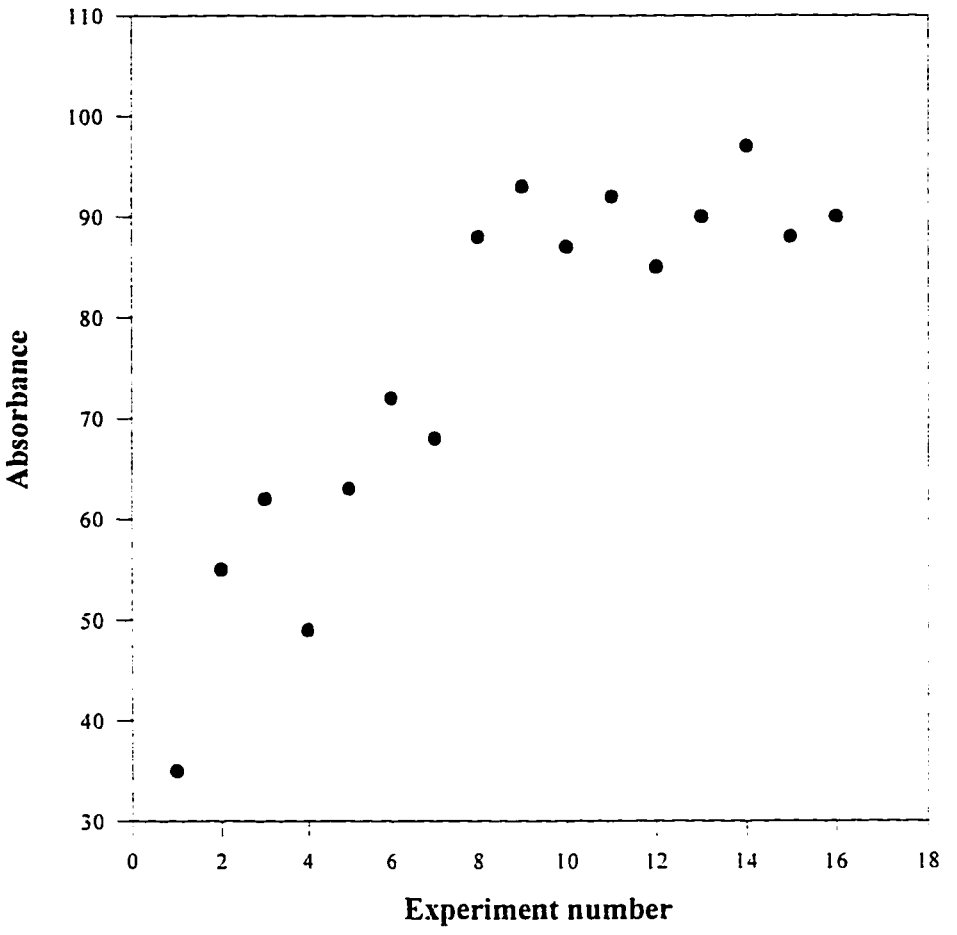


Figure 3.8 Progress of the response function of simplex for procanamide-HCl.

Table 3.9. Analysis of variance

Source of variation	DF[a]	SS[b]	MS[c]	F[d]	LS[e]
$[H_2SO_4]$ (A)	1	51.51	51.51	0.69	
[Ce(IV)] (B)	1	20889.68	20889.68	279.24	99.9%
Coil length (C)	1	447.01	447.01	5.98	99.5%
Flow rate (D)	1	51.51	51.51	0.69	
Sample volume(E)	1	20.48	20.48	0.27	
AB	1	898.88	898.88	12.02	99.9%
AD	1	187.21	187.21	2.50	
AE	1	31.21	31.21	0.42	
BC	1	396.21	396.21	5.30	95%
BE	1	80.01	80.01	1.07	
CD	1	202.01	202.01	2.70	
CE	1	334.11	334.11	4.47	95%
Residual[f]	19	1421.38	74.81		
Total	31	25011.20			

[a] Degree of freedom

[b] sum of squares (see text)

[c] mean square = SS/DF

[d] $MS_{effect}/MS_{residual}$ (see text)

[e] level of significance

[f] Residual contains higher order interaction plus small 2nd order interaction

to the instability of the oxidation products of procainamide at these levels.

3.4.3 Analytical Appraisals

The standard plot from which the calibration equation was calculated was obtained by running a series of procainamide HCl standards under the above conditions as shown in figure 3.10. Peak height versus procainamide HCl concentration was found to be linear over the range 100 to 600 ppm with the following calibration equation:

$$P.H. = -0.1837 + 0.2194 \times C;\ r = 0.999$$

where P.H. is peak height in mm; C is procainamide HCl concentration in ppm and r is correlation coefficient. The relative standard deviation for five injections of 250 ppm of the pure drug was 0.52%. Peak width at baseline was found to be 15 seconds thus a sampling rate of 250 samples per hour could be attained.

3.4.4 Application

The method was applied to the assay of procainamide HCl in the proprietary drugs Pronestyl tablets, Procainamide Durules tablets and Pronestyl injections with runs typically represented in peaks 10, 11 and 12 in figure 3.10. Results obtained indicated no interferences from excipients added in dosage forms, and almost the same degree of standard deviation of the results obtained by this method relative to those obtained by the British

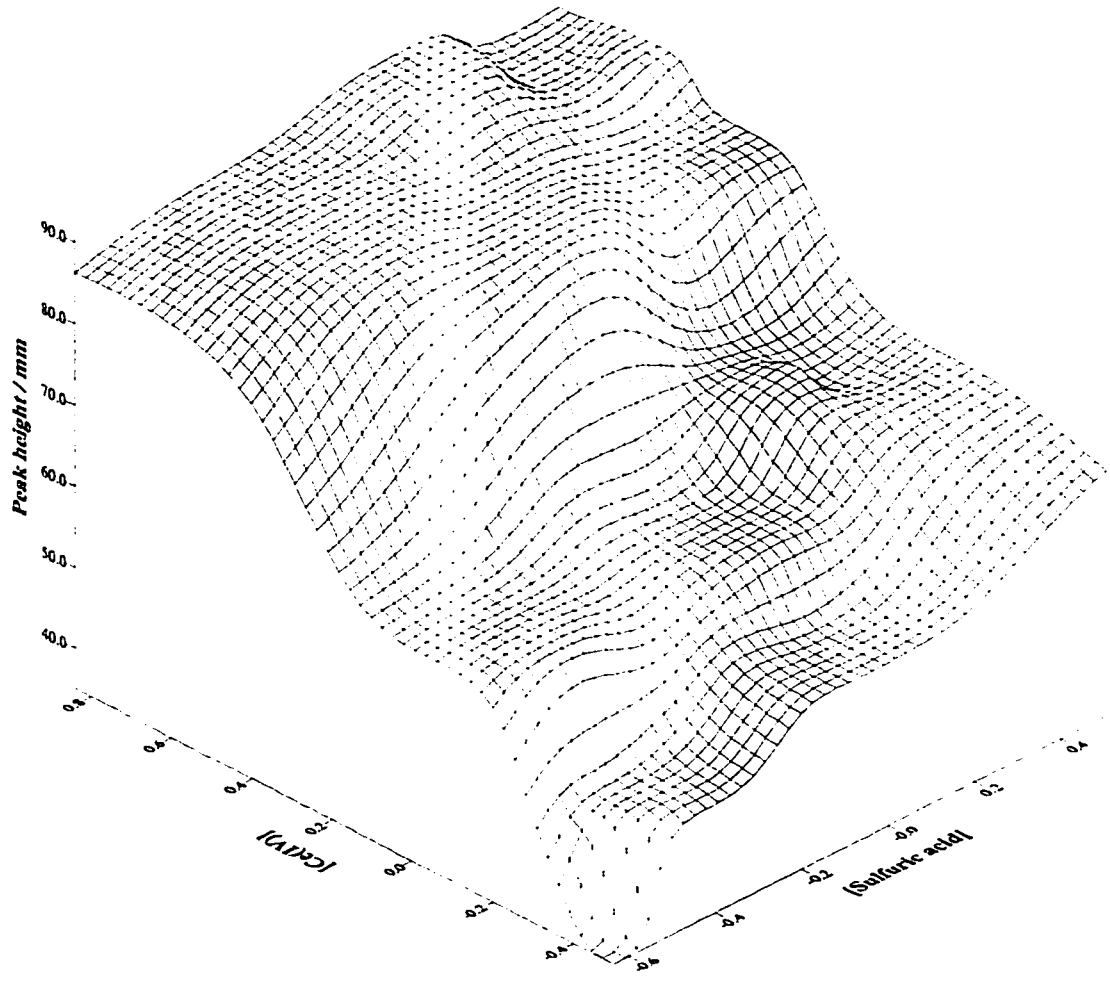


Figure 3.9 Pseudo-three dimentional plot of the peak absorbance vs. sulfuric acid and cerium(IV) concentration.

Table 3.10 Optimization obtained by the Super Modified Simplex program

Experiment #	$[H_2SO_4]$/M	[Ce(IV)]/mM	Coil length/cm	Peak height / mm
1	0.100	1.000	45	35
2	0.430	1.354	53	55
3	0.182	2.414	53	62
4	0.182	1.354	73	49
5	0.430	2.414	73	63
6	0.595	3.121	87	72
7	0.622	3.239	54	68
8	0.503	4.496	77	88
9	0.540	6.068	90	93
10	0.989	5.870	102	87
11	0.794	6.800	132	92
12	0.685	4.684	98	85
13	0.729	5.465	102	90
14	0.386	6.351	114	97
15	0.651	5.936	107	88
16	0.310	5.122	72	90

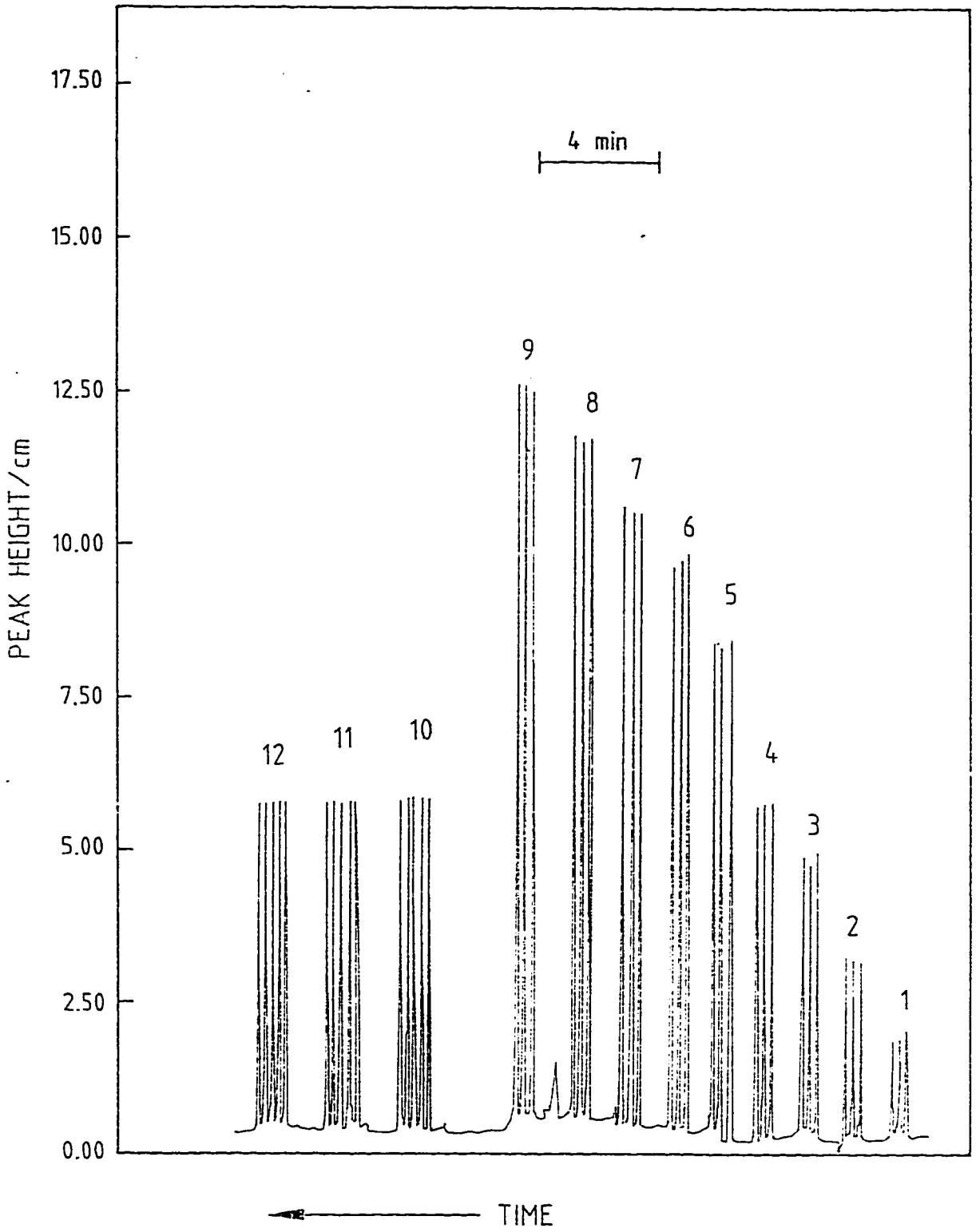


Figure 3. 10 Recorder tracing for FI measurements for series of standard procainamide-HCl solutions of (1) 99.0; (2) 148.6; (3) 205.2; (4) 247.6; (5) 346.7; (6) 403.3; (7) 452.8; (8) 502.3; (9) 601.4; (10) 250 (Pronestyl Tablets); (11) 250 (Pronestyl injections); (12) 250 (Procainamide Durules) ppm procainamide-HCl

Table 3.11. Results obtained by the FIA method and the BP method[17] for the analysis of procainamide HCl in proprietary drugs.

Drug	Supplier	Active material	Mean recovery ± SD(%)[a]		t[b]
			FIA-method	BP-method	
Pronestyl Tablet	Squibb,UK	procainamide 250 mg	100.2 ± 0.81	99.6 ± 0.91	1.7
Pronestyl injection	Squibb,UK	Procainamide 100 mg/ ml	99.9 ± 0.70	99.4 ± 0.92	1.6
Procainamide Durules tablets	Astra,USA	Procainamide 500 mg	100.3 ± 0.62	100.1 ± 0.85	0.72

[a] n = 5

[b] t theoretical value = 2.78, p = 0.05

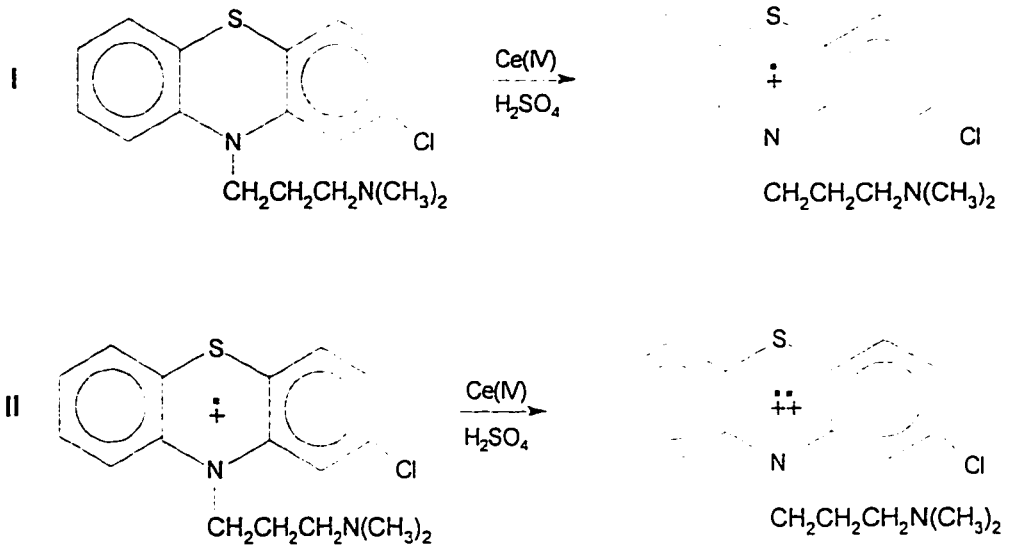
Pharmacopoeia method indicates the high degree of accuracy of the present method (see Table 3.11).

The method is superior to reported methods with respect to specificity and simplicity[69-72] when applied to the determination of procainamide HCl in drug formulations where no pre-treatment nor extraction process was necessary. Its advantages over the official BP method[77] are the significant shorter analysis time, the slightly higher precision and the suitability for micro analysis of the drug.

3.5 Assay of Chlorpromazine in Pharmaceutical Preparations*

Chlorpromazine hydrochloride is [3-(2-chlorophenothiazine-10-yl) propyl] dimethylamine hydrochloride, and is a member of a family of drugs commonly known as neuroleptic tranquilizers, and are used as sedatives, antihistamines, anti emetics and anesthetics. A variety of methods have been reported for the determination of chlorpromazine and have been recently reviewed[6]. Few FI methods have been proposed for the determination of chlorpromazine, including spectrofluorometric determination after photochemical derivatization[80,81] and a voltammetric method[82]. In the British pharmacopoeia[83] (BP) monograph, chlorpromazine, in syrup or in Tablet form, is treated by long extraction procedures and measurement of the resultant solution spectrophotometrically in the UV region.

* Suliman F. O., Sultan S. M., Talanta, 1994, 41, 1865.



Scheme 3.4 Reaction of chlorpromazine with cerium(IV)

The recent FI methods adopted for the assay of chlorpromazine and parent compounds[6,7] demonstrated the complexity of their reaction kinetics, mechanism and instability of products. However, the availability of useful computerized programs applicable to a variety of analytical methodologies led to the development of the present FI method well established by such chemometrical applications for the adoption of confident experimental conditions reliable for precise determination of this compound.

Ce(IV) is used as an oxidant for a FIA spectrophotometric method for the determination of chlorpromazine in sulfuric acid media. The reaction, as suggested earlier using a different inorganic oxidant[6], proceeds via formation of the monocation radical in one step and to the dication radical in the second step thus leading to unstable products and accordingly could be similarly suggested with cerium(IV) as shown in scheme 3.4. A single line manifold, where Ce(IV) in sulfuric acid solution was the carrier reagent was used.

The modified simplex method was used to select the optimum operating conditions, which were further investigated by response surface methodology (RSM) by using a 3^2 factorial design in order to check for the ruggedness of the method.

The response surface methodology (RSM), is a collection of mathematical and statistical techniques useful for analyzing relationships between several experimental variables and one or more responses[28,29]. These techniques involve the design of experiments, fitting of empirical or

theoretical models to the experimental data, and interpretation of the fitted response. Application of a properly designed experiment and adequate multifactor models may lead to better understanding of the response surface and safer prediction of the optimum operating conditions.

3.5.1 Optimization

The influence of the most critical variables on the magnitude of the peak absorbance and reproducibility of the results was studied carefully. The variables were divided into two groups, chemical variables (sulfuric acid concentration and cerium(IV) concentration) and system variables (flow rate, coil length and sample loop size). From preliminary investigations and a previous experience[6,7,84], the system variables were fixed to the optimum values shown in Table (3.12). The simplex method was then used to optimize the chemical variables separately. The first point conditions and response were fed into the computer and the program automatically calculated vertexes of the starting simplex as in Table (3.13). The simplex rapidly located the optimum region, and the optimization was halted after 15 experiments, since no further substantial improvement was expected. Therefore, the optimum chemical conditions for the assay of chlorpromazine were, sulfuric acid concentration 0.035 M and Ce(IV) concentration 3.8×10^{-3} M. The results obtained by the simplex manifested a successful application to the system with few number of steps required to attain maxima. From step 3 onwards, all points fall within the level of optimum conditions and for this reason, the phenomenon exhibited in figure

Table 3.12 Experimental ranges of variables used in optimization and optimum conditions obtained.

Variable	Range	Optimum
Flow rate	0-6.0 ml/min	4.85 ml/min
Coil length	45-200 cm	45 cm
Sample volume	50-250 µl	110 µl
$[H_2SO_4]$	0.01- 0.5 M	0.035 M
[Ce(IV)]	$1.0x10^{-4}$ - $1.0x10^{-2}$ M	$3.8x10^{-3}$M

Table 3.13 Simplex optimization of chemical variables

Experiment #	$[H_2SO_4]$ / M	[Ce(IV)] $x10^{-4}$ M	Peak absorbance
1	0.050	2.000	0.209
2	0.168	8.40	0.449
3	0.082	25.9	0.668
4	0.200	32.2	0.697
5	0.184	29.2	0.675
6	0.113	49.8	0.684
7	0.127	39.6	0.696
8	0.245	46.0	0.638
9	0.140	32.9	0.702
10	0.212	25.7	0.657
11	0.152	35.6	0.708
12	0.090	36.2	0.717
13	0.035	38.0	0.744
14	0.048	40.8	0.726
15	0.064	39.4	0.736

3.10 for the decrease of absorbance at higher Ce(IV) concentrations was not identified, simply due to the limited range of Ce(IV) concentration which is below the critical concentration that causes the decrease in absorbance. In this region the cerium(IV) effect is eliminated and it is only the sulfuric acid that plays a major role for the instability of the product and this is considered another development achieved.

3.5.2 Response Surface Modeling

A three level, two factor, full factorial design (with replications) study was carried out in the region of the simplex optimum. The analysis of variance (ANOVA) results are presented in Table (3.14) indicating that both factors sulfuric acid concentration and Ce(IV) concentration show significant interaction. The peak absorbance was then fit to a full second order polynomial model of the form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \qquad 3.3$$

where Y represents the estimated response, β_0 is an intercept parameter, β_1 and β_2 are linear parameters; β_{11} and β_{22} are quadratic parameters β_{12} is an interaction parameter; X_1 and X_2 represent sulfuric acid and cerium (IV) concentrations respectively. Table (3.15) gives the parameter estimates obtained by matrix least squares[28-30]. The level of significance shown in Table (3.15) is the level at which the null hypothesis for each parameter (H_o, $\beta = 0$) can be rejected, figure 3.11 is a pseudo-three dimensional plot of the

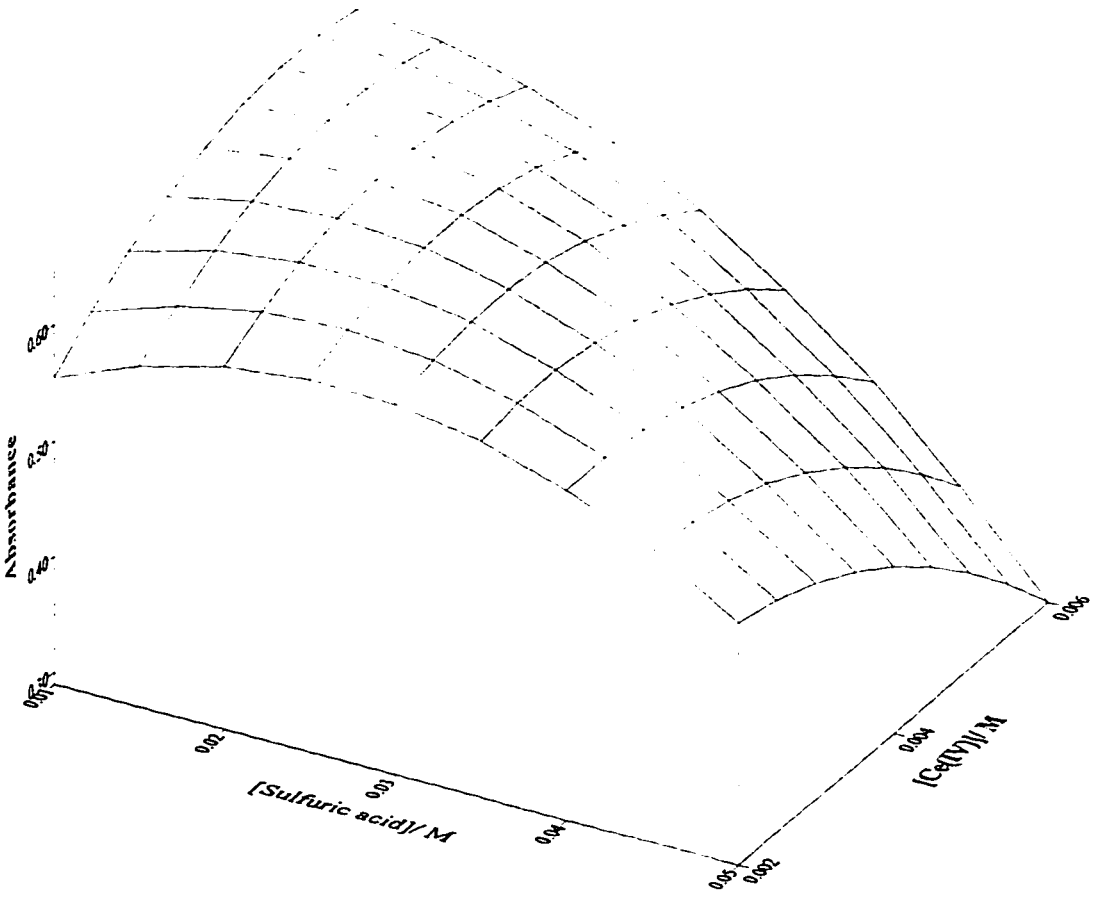


Figure 3.11 Response surface plot of peak absorbance vs. sulfuric acid and cerium(IV) concentration.

Table 3.14 Analysis of variance (ANOVA)

Source variation	Sum of squares	Degrees of freedom	Mean square	F-ratio *
$[H_2SO_4]$	0.155125	2	0.077563	443.8
[Ce(IV)]	0.016514	2	0.008257	47.2
Interaction	0.094551	4	0.023647	135.2
Residual	0.001573	9	1.7478×10^{-4}	
Total	0.267762	17		

* level of significance = 99.9%

response surface for the fitted model in the region of the optimum. It shows a diagonal ridge that decreases as both sulfuric acid and Ce(IV) concentrations are increased. Also decreasing both reagents results in a decrease in the peak absorbance. Both variables, sulfuric acid and Ce(IV), show a broad optimum at the intermediate level. Intermediate levels of 0.030 M sulfuric acid and 4.0 $x10^{-3}$ M cerium(IV) depicted from the simplex optimum conditions, clearly exhibited broad leveling in the pseudo-three dimensional plot which correlates very well with the results obtained by the simplex, thus confirming rigidity and tolerance of the small changes of variables of the simplex which is an indication of the successful location of optimum.

3.5.3 Cell Mean Plots

The cell mean plot of a factor is a graph of the measured response of each level of that factor averaged over all levels of the other factors. The relative importance of each factor can be obtained from this plot without fitting a model to the data. The interaction between two factors can be inferred from the cell mean plots of one factor at the different levels of the second factor[85,86]. The main effect cell mean plots are greatly dependent on the significance of interaction between factors. If the interaction between factors is insignificant, then increasing the level of a factor will result in the same behavior at all levels of other factors.

Figure 3.12 shows the effect of sulfuric acid concentration at different Ce(IV) concentration on the peak absorbance. At low level of Ce(IV), increasing the sulfuric acid concentration causes the peak absorbance to

Table 3.15. Regression Analysis

Variable	Parameter *	Estimate	Level of significance(%)#
Intercept	β_0	0.26299	99.9
Sulfuric acid	β_1	14.8333	98.0
	β_{11}	-207.71	97.5
Cerium(IV)	β_2	120.437	90.0
	β_{22}	-9833.33	85.0
Interaction	β_{12}	-1881.25	99.5

* see equation 3.3

level at which null hypothesis can be rejected.

pass through an optimum at the middle level of the factor. At intermediate and high levels of Ce(IV), the peak absorbance decreases with increasing sulfuric acid concentration.

From a theoretical point of view the above observations can be interpreted as follows. The redox potential of Ce(IV) increases with increasing the acid concentration resulting initially in an increase in the amount of the oxidation product and hence in the peak absorbance. However, the rate of degradation of the radical cation which was believed to be the product of oxidation of this compound increases with increasing the acid and oxidant concentrations resulting in a decrease in peak absorbance. This is clearly manifested in figure 3.12, with a sharp decrease of peak absorbance at high sulfuric acid and Ce(IV) concentration. At this level and with respect to the kinetics of the first step reaction (scheme 3.4), an increase in Ce(IV) and sulfuric acid concentrations would result in an appreciable increase in the monocation radical intermediate which in turn acts as a reactant for the second step in the presence of excess Ce(IV) and sulfuric acid, thus rendering the monocation radical a highly unstable intermediate leading to a sharp decrease in the response. This clearly demonstrates that the choice of Ce(IV) as a milder oxidant than molybdate and dichromate used for the assay of this drug in the previous methods[6,7], is a great advantage and a significant development reflected in higher precision and more acceptable reproducibility. The relative standard deviation (RSD) calculated was found to be less than 0.7% in the present method and > 1.8% in the molybdate and the dichromate methods. Therefore , one should carefully adjust the concentration of both reagents

to maximize the amount of the oxidation product and to minimize the degradation of this product.

3.5.4 Analytical Appraisal

A series of standard solutions containing chlorpromazine in the range 50-200 ppm were injected in triplicate with a typical run as shown in figure 3.13. Peak height with an average relative standard deviation of 0.70% for five repeated injections indicate high reproducibility. The plot of absorbance (A) *versus* chlorpromazine concentration (C) was linear over the concentration range 50-200 ppm. A weighted regression line was plotted, which resulted in the calibration equation: $A = -0.17253 + 4.8327 \times 10^{-3} C$; with a correlation coefficient (r) of 0.999. A sample frequency of 120 h^{-1} could be obtained, and this was determined by measuring the peak width at the baseline, which was found to be 30.0 s and 3.25 s at 60% of the peak height indicating minima! dispersion.

The method was applied to the determination of chlorpromazine in proprietary drugs, Largactil Tablets, with runs typically represented in peaks (7) and (8) in figure 3.13. The same batches were analyzed by the BP(85) method and the results were statistically compared as shown in Table (3.16). The present method showed almost the same degree of accuracy of the BP method, and the results obtained indicated no interferences from excipients added in dosage forms.

The sequential optimization of variables validated the flow injection method with better confidence and reliability of the results obtained indicated by higher precision, excellent reproducibility with higher

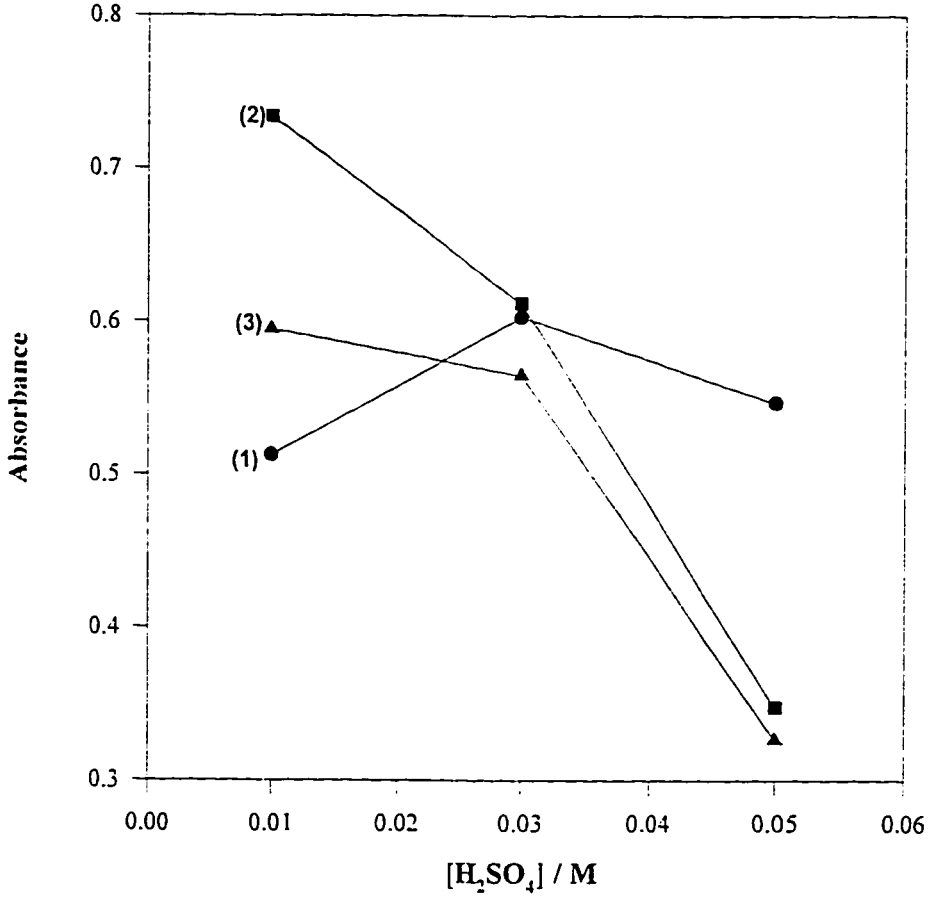


Figure 3.12 Cell mean plot of sulfuric acid concentration at different levels of cerium(IV) concentration of (1) 0.002 M; (2) 0.004 M; (3) 0.006 M.

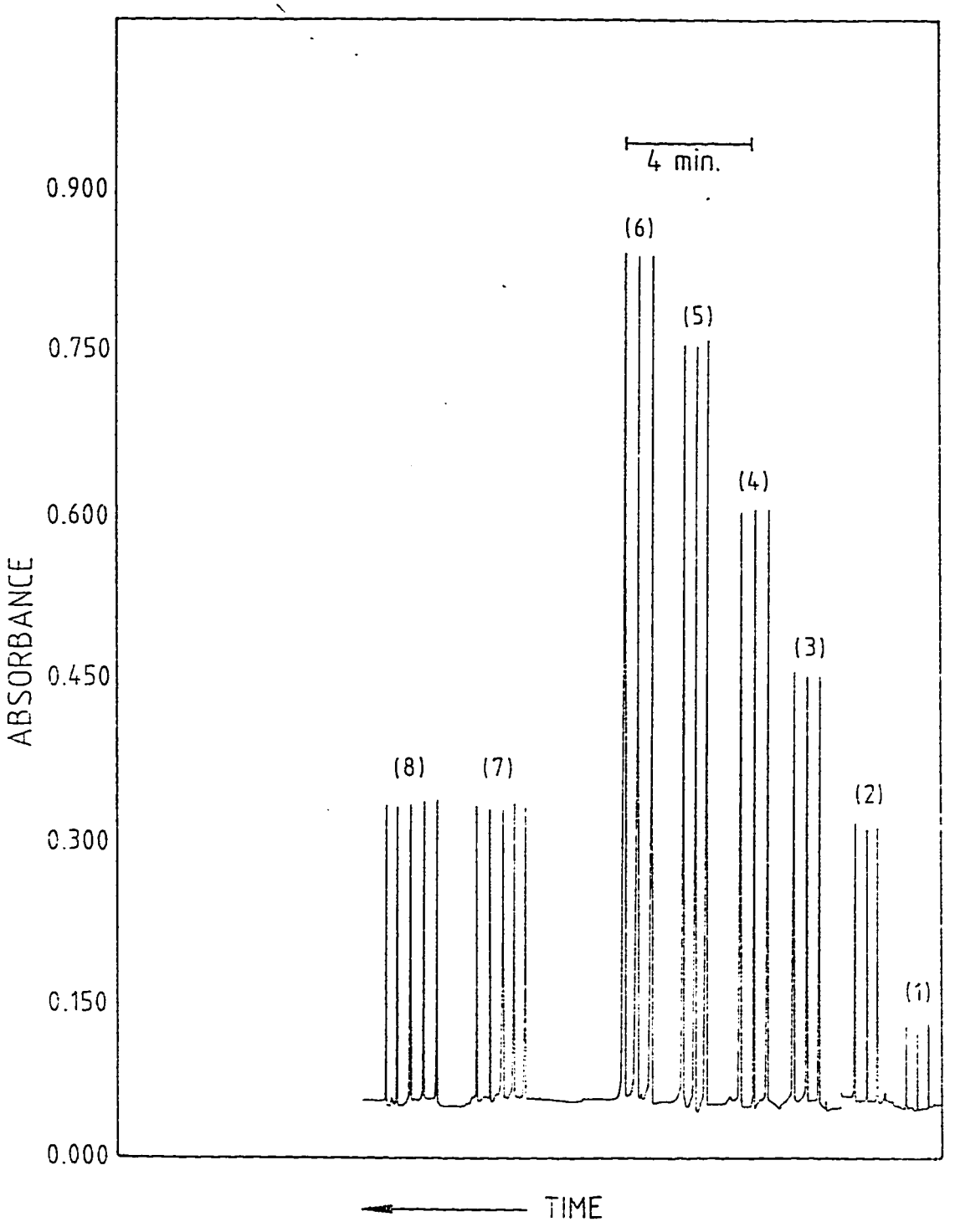


Figure 3. 13 Recorder tracing for FI measurements for series of standard chlorpromazine solutions of (1) 50; (2) 100; (3) 120; (4) 150; (5) 180; (6) 200; (7) 100 (Largactil Tablets 100 mg); and (8) 100 ppm chlorpromazine (Largactil Tablets 25 mg).

Table 3.16. Results obtained by the FIA method and the BP(83) method for the analysis of chlorpromazine in proprietary drugs

Drug	Supplier	Contents/mg	Mean recovery ± SD(%)[a]		t[b]
			FI-method	BP-method	
Largactil Tablets	Specia, France	Chlorpromazine 100	100.1 ± 0.80	101.2 ± 0.70	1.6
Largactil Tablets	Specia, France	chlorpromazine 25	99.6 ± 0.86	100.5 ± 0.50	1.8

[a] n = 5

[b] t theoretical value = 2.78, n = 5

Table 3.16. Results obtained by the FIA method and the BP(83) method for the analysis of chlorpromazine in proprietary drugs

Drug	Supplier	Contents/mg	Mean recovery ± SD(%)[a]		t[b]
			FI-method	BP-method	
Largactil Tablets	Specia, France	Chlorpromazine 100	100.1 ± 0.80	101.2 ± 0.70	1.6
Largactil Tablets	Specia, France	chlorpromazine 25	99.6 ± 0.86	100.5 ± 0.50	1.8

[a] n = 5

[b] t theoretical value = 2.78, n = 5

sensitivity and wider range of drug concentration when compared to the previous reported methods.

3.6 Assay of Perphenazine in Drug Formulations

Experimental design is a powerful tool for optimization, ruggedness testing and modeling of chemical analysis procedures usually employed in order to understand the relationship between the experimental factors and their corresponding responses, as well as to determine the optimum operating conditions at which small changes of these factors can be tolerated. Screening designs, such as two level factorial or fractional designs are very useful for ruggedness testing and for the elimination of insignificant factors from optimization in which only highly interacting factors are to be considered[87-91]. Simplex method has proven successful for the optimization of chemical reactions in flow injection procedures[87-89,92-93] and has proven to be an efficient technique when four or more interacting variables are involved. Experimental design together with response surface modeling can be used to understand the manner in which the system variables affect the response function[28,29]. In response surface modeling, the system is represented by an empirical equation derived by fitting coefficients to preselected equation forms using system response data. Typically second order polynomial models with cross terms are used.

Simplex optimization data points are usually irregularly spaced and clustered around the optimum, and when used in response surface modeling, only the region close to the optimum will be efficiently characterized. Surface mapping by simplex in FIA requires a fully automated technique so that reasonable number of replicated experiments can be obtained under different chemical and flow situations within a short period of time[94]. These chemometrical optimization approaches are demonstrated here for the development of a method for the assay of perphenazine in drug formulations using FIA technique. In the method cerium(IV) was used as an oxidant in sulfuric acid media. The colored product of the oxidized form of perphenazine is followed spectrophotometrically at 525 nm utilizing a double-line manifold.

Perphenazine, 4-[3-(2-chloro-1 OH-phenothiazine-10-yl) propyl]-1-piperazineethanol belongs to the phenothiazines family of drugs. It is used as an antihistamine, antiemetic, and for the treatment of Parkinson disease. Recently[95], perphenazine has been determined by FIA but with poor sensitivity, reproducibility, and very limited linear dynamic range. In the method described[95], the system and chemical variables were optimized using a univariate procedure. A very high flow rate was selected for the method to attain a sample throughput of 300 samples per hour at the expense of sensitivity and reproducibility.

A systematic optimization study is employed to set the best conditions for the determination of perphenazine. Besides the optimization study, it is aimed to model the response surface in an attempt to correlate

the effect of the experimental factors and the kinetics of the reaction to the response function.

3.6.1 Optimization

Super modified simplex procedure[26] was initially utilized for the optimization of five parameters: sulfuric acid concentration, cerium(IV) concentration, flow rate, coil length, and sample loop size. The criterion on the basis of which the performance of the analytical system can be evaluated so that the optimum operating conditions can be obtained, was selected to include the sample throughput and the sensitivity in one expression similar to that given in equation 3.1 as follows:

$$R = \frac{A_{exp}}{A_{base} \cdot t_b} \times 100 \qquad 3.4$$

Where R is the response function, A_{exp} absorbance of the peak absorbance at the peak maximum for a set of conditions, A_{base} is peak absorbance for the reference conditions, t_b is the peak width at base line. The response function is related to the sensitivity by the term A_{exp}/A_{base} and to the sample through by t_b. The ranges of the five experimental variables investigated are given in Table (3.17).

The progress of simplex is given in figure 3.14 showing a gradual improvement in the response function and clear leveling towards maximization. The simplex results given in figure 3.14 were obtained by injecting 200 ppm drug standard solution. A total of 29 experiments were

Table 3.17 Experimental ranges of variables used in optimization together with optimum operating conditions obtained.

Factor	Range	Simplex Optimum	Experimental Design Optimum
[Sulfuric acid] A,M	0.010 - 0.500	0.170	0.152
[Cerium (IV)], B, mM	0.10 - 10.0	2.13	1.76
Flow rate, C, ml/min	0 - 6.00	2.56	3.31
Coil length, D, cm	45 - 200	52	65
Loop size, E, μL	110 - 200	136	138

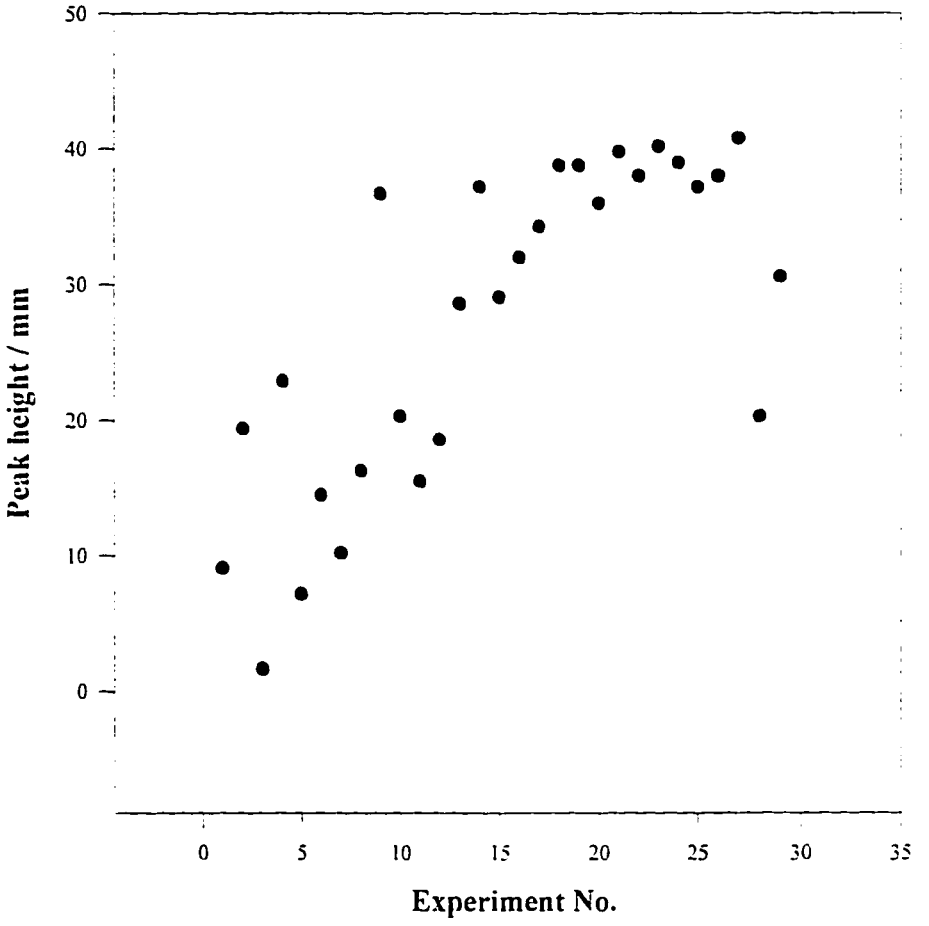


Figure 3.14 Response function progress of the simplex for the perphenazine system.

needed to decide upon the optimum conditions. The optimum conditions were obtained by calculating the average of the four sets of conditions that give rise to the top four responses. The maximum response functions obtained are more or less identical, and range between 39.8 and 40.8. Also the set of conditions generated these maximum responses vary within a very narrow range. Therefore, the optimum operating condition obtained by the simplex technique for the determination of perphenazine are, 0.170 M sulfuric acid, 2.13 x 10^{-3} M cerium(IV) concentration, flow rate of 2.56 ml/min, coil length of 52 cm and sample loop size of 136 μL.

The response surface was then mapped and explored by the aid of a rotatable five factor central composite design[28,29]. The central composite design, introduced by Box and Wilson[96], consists of a 2^k factorial or fractional factorial design augmented by 2k axial points and n_o center points, where k is the number of factors. The five central composite design is composed of 42 experiments plus 6 center experiments, so that a total of 48 experiments was performed. The coded levels of the variables together with their real experimental values are given in Table (3.18). The center point represented the set of optimum conditions obtained by the simplex, except for the coil length, which was changed from 52 cm to 75 cm in order to simultaneously cover a broad region of factor space, within the factor limit and to maintain the desired properties of the design. A software package was used to generate the full design in two blocks and to randomize the time order of the experiments within each block. All the experiments of the design were performed within one day.

Table 3.18 Coded levels of the central composite design and their respective experimental set-up.

Factor	Central composite design levels				
	2.4378	1	0	-1	-2.4378
$[H_2SO_4]$, A M	0.285	0.220	0.170	0.120	0.05
[Ce(IV)], B mM	4.21	3.03	2.13	1.23	0.05
Flow rate, C ml/min	4.65	3.47	2.56	1.65	0.47
Coil length, D cm	105	88	75	62	45
Loop size, E µL	164	148	136	124	110

3.6.2 Response Surface Modeling

Response surface methodology (RSM) was used to assemble the model that describes the way in which the variables are related and the way in which they influence the response function. The form of the relationship between the response function and the experimental variables is initially assumed to be unknown. Therefore, the data obtained from the set of conditions employed by the central composite design were fit to the following parametric equation (full second order polynomial)

$$R = \beta_o + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \qquad 3.5$$

where R is the response (given by equation 3.4), β_o is the intercept, X_i's are the five main variables, β_i is linear parameter, β_{ii} is quadratic parameter, β_{ij} represents interaction parameters. Table (3.19) gives the estimates of the 21 parameters, contained in equation 3.5, obtained by matrix least squares[29,30].

The adjusted correlation coefficient of the regression (R^2) was found to be 0.956. The significance of regression was tested by the F-ratio of the mean square due to the factors divided by the mean of squares due to the total error, and was found to be highly significant. The analysis of the residuals from the regression model and also the lack of fit test, revealed that the second order polynomial model, tentatively assumed, was an adequate description of the surface over the region studied.

Table 3.19 Multiple regression analysis

Regressor variable	Parameter	Parameter estimate	Significant level, %
Intercept	β_o	37.9350	100
Sulfuric acid, A	β_1	-0.2630	86
AA	β_{11}	-1.2006	99.5
	β_2	-0.5423	99.6
[Cerium(IV)], B	β_{22}	-1.8316	100
BB			
	β_3	4.4814	100
Flow rate, C	β_{33}	-2.3700	99.9
CC			
	β_4		
Coil length, D	β_{44}	-0.9065	99.5
DD		-0.5780	99.9
	β_5		
	β_{55}		
Loop size, E		0.5126	99.3
EE	β_{12}	-0.7631	99.5
	β_{13}		
AB	β_{14}	0.7844	99.9
AC	β_{15}	-0.2844	82.7
AD	β_{23}	0.2594	78.7
AE	β_{24}	-0.01563	6.0
BC	β_{25}	-0.8094	99.5
BD	β_{34}	-0.2781	81.8
BE	β_{35}	0.3469	90.1
CD	β_{45}	0.003125	1.2
CE		0.06563	24.7
DE		0.4719	71.7

3.6.3 Response Surface Plots

Response surfaces for all two factor interactions were produced using the second order polynomial model. All plots were generated as a function of two factors at a time by holding the other factors constant at the zero level. Only few plots, specially those for factors with considerable interaction, are shown here.

Figure 3.15 shows the response surface as a function of the sulfuric acid concentration and cerium(IV) concentration, other factors are at their zero levels. Both factors exhibit significant main effects as well as highly significant interaction effects (Table 3.19). It is evident from figure 3.15 that a broad optimum is obtained at the middle levels of both factors. Moving away from the optimum in the direction of high acid concentration and low cerium(IV) concentration, a sharp decrease in the response is observed. A similar trend is observed when the cerium(IV) concentration is at the high levels and at low levels of acid. When one of the factors is at high level whereas the other factor is at low level, a decrease in response is also observed but it is not as rapid as in the previous two cases. The oxidation of perphenazine with cerium(IV) was very fast producing a red colored product that absorbs at a wavelength of maximum absorbance at 525 nm. The reaction, as suggested earlier, using another oxidant and for other phenothiazines,[6,95] involves a first step, in which the perphenazine is oxidized to a monocation radical, which is the red colored absorbing species. In the second step, the monocation radical consumes another

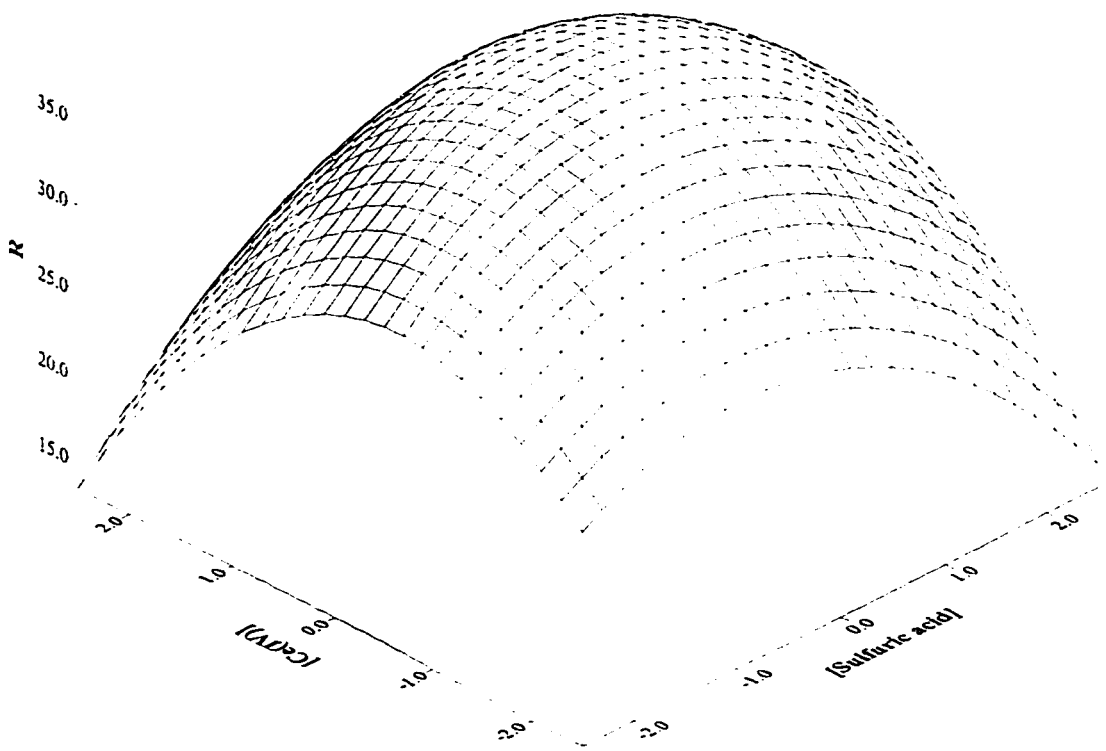


Figure 3.15 Surface plot of response function vs. sulfuric acid and cerium(IV) concentration; all other factors are at their zero levels.

oxidant molecule to produce dication diradical which is highly unstable and reacts very fast to produce colorless products. It is clear that just enough acid is needed to raise the redox potential of an appropriate cerium(IV) concentration to the level which will just oxidize the drug to the monocation radical. This explains the highly significant interaction between these two chemical factors. At high acid concentrations but low cerium(IV) the decrease in the response function can be attributed to the instability of the product. At low levels for both factors, sulfuric acid and cerium(IV), the decrease in response is due to the fact that the reductionpotential of the cerium(IV) is not attained to produce maximum amount of the product.

Figure 3.16 shows the response surface due to the second factor (cerium(IV)) and the third factor (flow rate) by keeping all other factors at constant zero level. The response function decreased significantly at the low levels of both factors, and slowly when both factors are at their highest levels. However, when cerium(IV) is at its low levels the response function increases rapidly with flow rate and then becomes independent of it, whereas at higher levels of cerium(IV), the response function increases with flow rate, reaching an optimum and finally decreases slowly. At low levels of flow rate the response increases rapidly with cerium(IV) concentration passing through an optimum around the middle levels of the experimental design and then it starts to decrease. Both factors, cerium(IV) and flow rate shows highly significant main effects and interactions when multiple regression analysis was applied (see Table 3.19). Theoretical and experimental studies[97] demonstrated the presence of an inverse relationship between the flow rate and peak width, or baseline to baseline

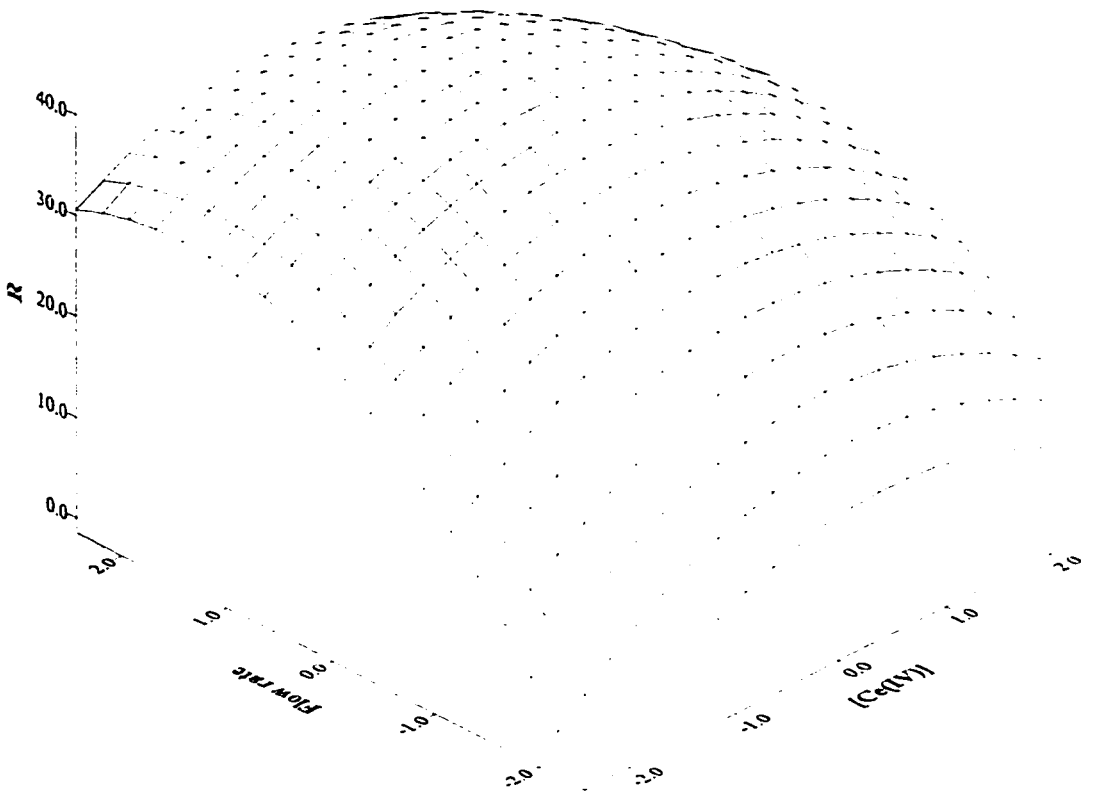


Figure 3.16 Surface plot of the response function vs cerium(IV) and flow rate; all other factors are kept constant at their zero levels.

time. Therefore, the decrease of response at low levels of flow rate and cerium(IV) can be attributed to dispersion as well as insufficient oxidation of the drug . At high flow velocities the dispersion is also decreased, resulting in improved peaks the height of which becomes independent of flow rate at all levels of cerium(IV) concentration. The decrease in response at high cerium(IV) levels is probably due to the fact that higher concentration of cerium(IV) retards the reaction rate and lower peak height is therefore the result.

Figure 3.17 shows the response surface as a function of sample loop size and cerium(IV) concentration, keeping the other factors at their zero level. At high levels of cerium(IV) the response is decreased significantly due to the retardation of the reaction rate, same reason as above. At low levels of cerium(IV) the response function increases with the loop size increase owing to the fact that the effect of decrease of dispersion becomes a predominant factor. The decrease of response at low concentration of cerium(IV) can be attributed to the insufficient oxidation of the drug as above. Also it can be inferred from this figure that the response is less sensitive to changes in loop size than to cerium(IV) concentration.

Figure 3.18 shows the response surface as a function of coil length and loop size and all other factors are kept constant at their corresponding zero levels. These two variables are used to define the parameter, α[31], which is the ratio of the sample zone length prior to injection, to the length of the reactor. Dispersion increases with decreasing α, either by increasing the coil length or decreasing the loop size. At high levels of coil length and low levels of sample loop size, dispersion predominates, as α is minimal at

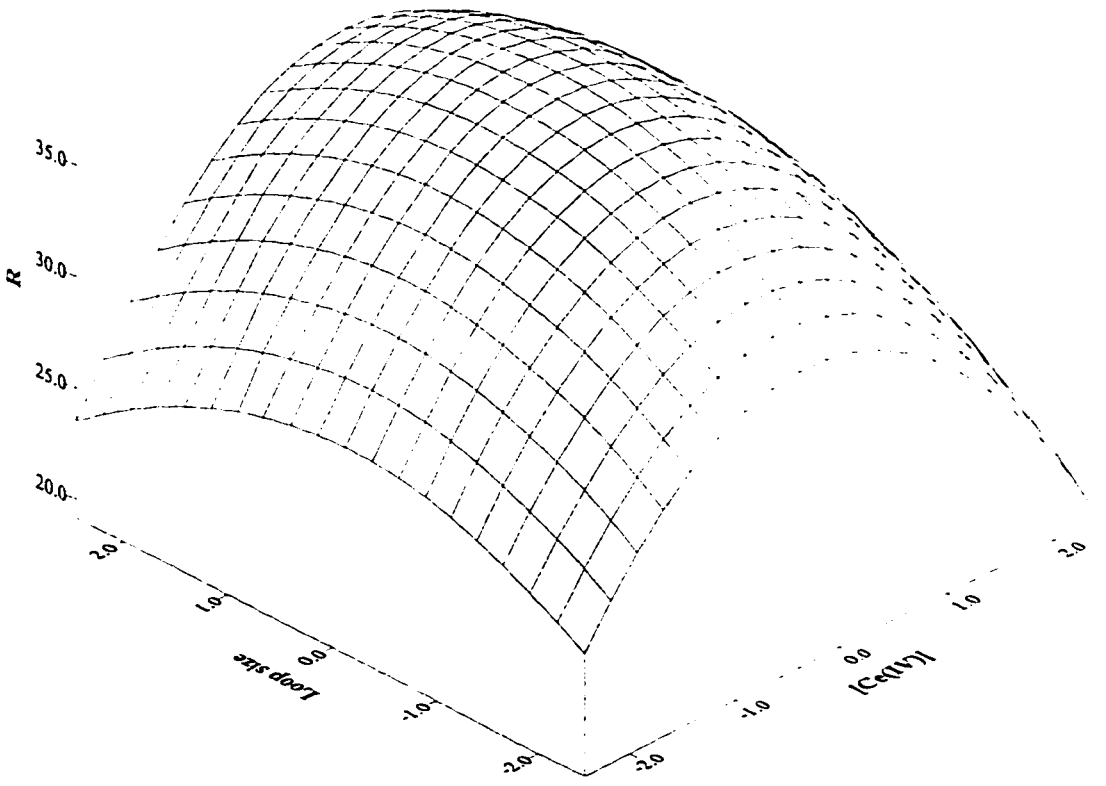


Figure 3.17 Surface plot of the response function vs. cerium(IV) and loope size; all other factors are kept constant at their zero levels.

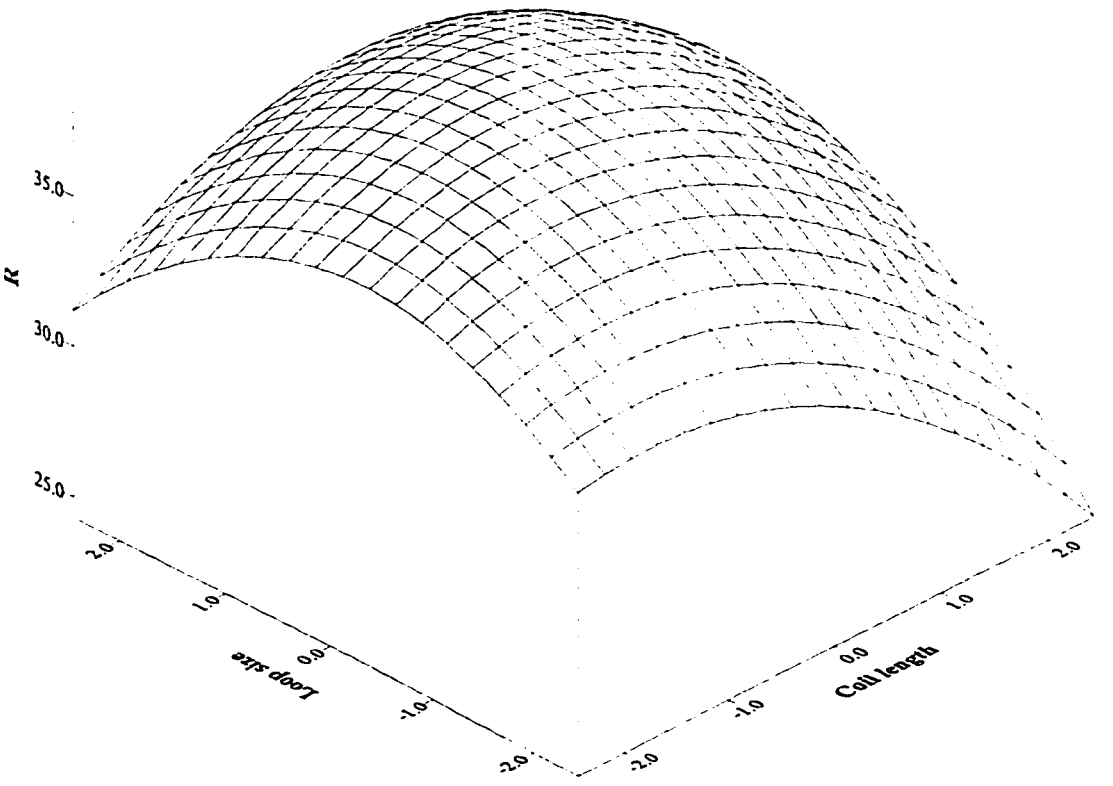


Figure 3.18 Surface plot of the response function vs. coil length and loop size; all other factors are kept constant at their zero levels.

these levels, resulting in a sharp decrease in the response. At low levels of coil length, the peak height increases initially up to the middle level beyond which it becomes independent of the loop size. On the other hand peak width increases slowly with loop size resulting in a decrease in response function for longer loop size. The increase in response at longer coil length is governed mainly by the sharp linear increase of peak height when the sample loop size is increased , whereas the peak width was found to be important only at high levels of loop size. The preference to shorter coil lengths is obvious from this figure thus confirming the simplex results where the optimum coil length was found to be 52 cm (-1.76 coded levels).

Investigation of the other response surfaces (not shown here) revealed the presence of a broad simple optimum around the simplex conditions (zero levels of the design). Preference to high flow rate is also observed in most of the cases, and also to shorter coil lengths. At high flow rate and short coil lengths minimum dispersion is attained, which is reflected in increased peak height and decreased peak width and hence an improved response.

3.6.4 Canonical Analysis

Canonical analysis[28] is performed by translating the response function from the origin where all factors are at zero levels to the stationary point, then the response function is expressed in terms of new variables the axis of which correspond to the principal axis of the response surface. The stationary point of the response is determined by differentiating the fitted regression model with respect to each factor simultaneously, equating to

zero, and then solving. For our model, the stationary point was located at the coordinates (considering coded levels) - 0.450, - 0.412, 1.043, - 0.760, and 0.0567, for sulfuric acid, cerium(IV), flow rate, reaction coil length and sample loop size, respectively. The predicted response at the stationary point was 40.802 which is in close agreement to the values obtained by the simplex method. The canonical form of the response function was found to describe a simple clear maximum, as all of the canonical coefficients were found to be of negative sign, and is given by the following equation:

$$\hat{R} = 40.802 - 2.60109\,W_1^2 - 1.90831\,W_2^2 - 0.96676\,W_3^2 - 0.86427\,W_4^2 - 0.4028\,W_5^2 \quad 3.6$$

where $\hat{R}$ is the estimated response, and W_1, W_2, ..., W_5 are the canonical variables.

3.6.5 Analytical Application

The optimum operating conditions obtained by the simplex procedure and those obtained from the stationary point of the experimental design are shown in Table (3.17). Series of standard perphenazine solutions were run using the set of conditions obtained by the super modified simplex optimization procedure, and the stationary point conditions one at a time. Peak absorbance versus concentration was found to be linear between 50-500 ppm for both methods, and almost two-fold improvement in the linear range as compared to the previous study(95). The calibration equations

Table 3.20 Results of statistical analysis of synthetic samples containing perphenazine using optimum conditions obtained by the simplex method compared by those of the experimental design stationary point.

Species added *	Recovery ± SD (%)@		t#
	Simplex method	Experimental design method	
Starch	99.98 ± 0.57	99.11 ± 0.46	0.21
Glucose	97.74 ± 0.50	95.46 ± 0.59	0.56
Lactose	101.84 ± 0.48	100.18 ± 0.58	0.40
Maltose	101.54 ± 0.75	99.98 ± 0.77	0.53

obtained for the simplex optimized method and for the experimental design stationary point are respectively as follows:

$$A = -0.02812 + 1.916 \times 10^{-3} C, \quad R^2 = 0.998$$

$$A = -0.034 + 2.233 \times 10^{-3} C, \quad R^2 = 0.997$$

Where A is the peak absorbance and C is the concentration of perphenazine in ppm. The relative standard deviation was found to be 0.74% and 0.77% , using five repeated determinations of 200 ppm drug, when the simplex and experimental design methods set of conditions were performed, respectively. The stationary point of the experimental design was found to be characterized by a higher sampling frequency, 190 h^{-1} compared to the simplex depicted method of only 120 h^{-1}.

The tolerance of the two sets of optimum operating condition to excipients which can be present in a typical pharmaceutical preparation containing perphenazine was investigated. Synthetic solutions of 200 ppm perphenazine in the presence of 2000 ppm interfering compounds, such as starch, glucose, lactose and maltose, have been analyzed by the two optimum conditions. The percent recovery, standard deviation (SD) and the student t-test values are given in Table(3.20). The student t-test values reveal that a similar degree of accuracy is obtained by the two set of conditions. Foreign compounds expected to be present in drug formulations show minimum or no interference in both cases.

This work demonstrates the inevitable need for systematic optimization of system variables in order to obtain and propose a rapid, sensitive and reproducible quantitative procedure. The super modified simple procedure successfully attained optimum conditions which were further confirmed by experimental design techniques. Response surface modeling helped in understanding the effect of each factor on the response and to correlate that to the kinetics of the chemical reaction.

CHAPTER FOUR

4. Sequential Injection Analysis

Sequential injection (SI) is a versatile computer controlled technique that has been recently introduced by Ruzicka and Marshall[19] with so far only a limited number of publications. In sequential injection (SI) technique, a selector valve equivalent to injection valve in flow injection (FI) is used for precisely measured volumes of carrier, sample and reagent solution to be aspirated into a holding coil (HC) which are then propelled by a flow reversal into a detector. By this technique, consumption of valuable reagents, drugs, etc., could be minimized by injecting small amounts using the selector valve rather than being pumped continuously as in the case of FI. With the SI technique a number of complicated operations such as dilution, injection of additional reagents, such as acids, and recalibration could all be executed easily and smoothly with the selection valve which is controlled by a simple computer program.

It is apparent that the sequential injection (SI) technique is still an infant and its full versatility and capacity are yet to be explored. SI technique involves one pump, one valve, and a single channel making it simpler than the conventional flow injection FI technique. Once constructed, the mechanical flow system of a SI - apparatus does not have to be reconfigured for different injection volumes, reaction times or mutual zone dispersion, because these parameters can be controlled directly by

changing volumes of flow reversals and forward flow rate from the computer key-board. (20)

Cumbersome jobs which usually bother experimenters, can be performed with some ease using SI. For instance, the determination of stoichiometries and formation constants of complexation reactions involves the preparation of a large series of solutions at carefully adjusted conditions and precisely repeating the procedure until acceptable results are obtained. When a valuable and an expensive reagent is to be used, the above operations become difficult to repeat and usually reporting of poor data is the final result.

4.1 Determination of Phenothiazines

The SI technique was used for the determination of trimeprazine and perphenazine by spectrophotometry. A colored complex was formed between the drug and palladium(II) which was monitored at 515 nm for trimeprazine and at 560 nm for perphenazine. It is interesting to note that both reagents, palladium and the sample (drug) were injected, thus consuming minimum reagent.

The SI technique was also used for the investigation of reaction stoichiometry utilizing the Job's plot method(45). The SI method was applied by sequentially aspirating the drug solution followed by the palladium(II) solution. A volume of carrier solution was then aspirated. The carrier volume was selected to allow optimal mixing upon flow reversal

towards the detector. The palladium(II)-to-drug mole ratio was varied, for the Job's plot analysis, by changing the aspiration timing of equimolar solutions of palladium(II) and the drug into the holding coil. The total volume of the two reagents was held to a constant value of 352.8 μl for each run. The ability of the SI apparatus to vary the aspiration time of a reagent conveniently with millisecond precision is considered a great advantage and a revolution in FI methodology, having the potential to solve many research problems.

4.1.1 Chemical System

The determination of the drugs is based on the complexation reaction of palladium(II) with trimeprazine or perphenazine in hydrochloric acid media which was extensively investigated and found to be feasible in a specific pH range. Other phenothiazine drugs were reported to form complexes with palladium(II) of unusual stoichciometry resulting in doubtful rationalization of the complex structures.(100-106) However, the stability of the complexes formed favored this method and was found to be more advantageous over the previous methods involving oxidation and in which a highly unstable radical product is monitored for quantification.(6,7,93,95)

The nature of the interaction between the palladium(II) and the drugs under investigation was believed to involve protonation of the drug followed by hydrogen bonding between the protonated nitrogen and one of

the chlorine atoms on the palladium[100-106]. Complexes of the type ML_2X_2 and MLX_3 have also been reported[106], (where M = Pd, L = phenothiazine ligand, X = Cl).

Preliminary investigations during this work revealed that in acidities higher than 1.0×10^{-4} M HCl, another absorbance maximum at a longer wavelength than that of the complex was observed. This could be attributed to dissociation of the complex and the appearance of the oxidized form of the phenothiazine. It was therefore, necessary to optimize the chemical conditions prior to the quantitative measurements as outlined below.

4.1.2 The Trimeprazine System

The purple colored complex formed was found to occur very fast with a peak maximum at 515 nm and remained stable for more than two days.

The effect of acidity on the complex was investigated for 160 ppm trimeprazine and 1.14×10^{-3} M palladium(II) between 3.51 to 6.0 pH range as in figure 4.1. The complex was found to be stable in a wide range of HCl concentration i.e., between 4 and 5.5 pH range.

The effect of palladium concentration on peak absorbance was studied for 50 ppm, 200 ppm and 400 ppm trimeprazine at pH 4.7 using palladium concentration in the range of 1.0×10^{-4} to 6.0×10^{-3} M as shown in figure 4.2. It can be observed that the increase in absorbance was not significant beyond 5.5×10^{-3} M palladium.

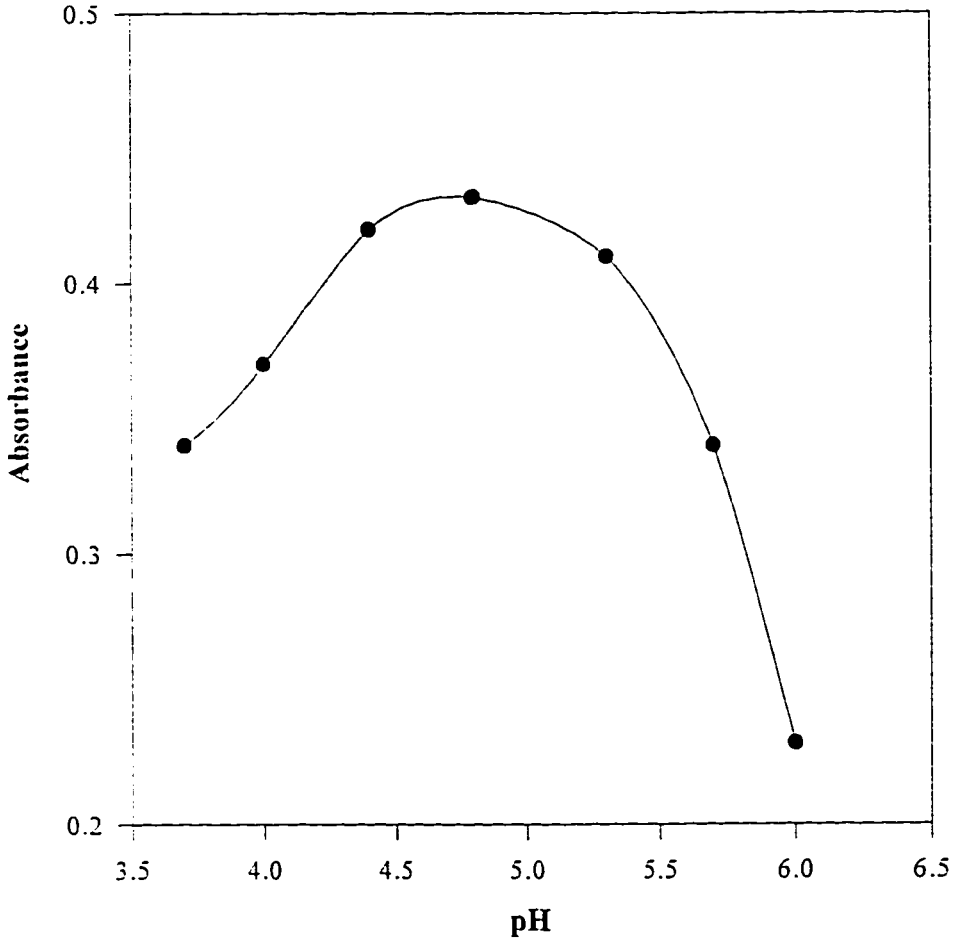


Figure 4.1 Effect of pH on complexation of 1.14×10^{-4} M Pd(II) with 160 ppm trimeprazine

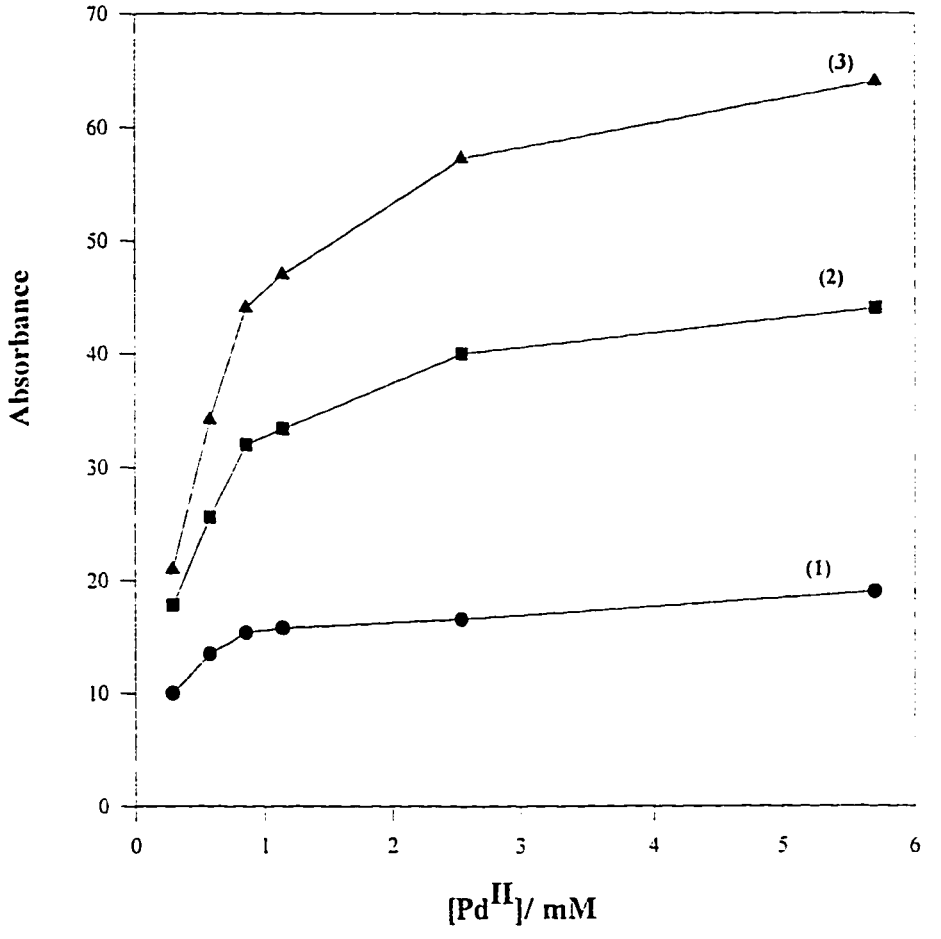


Figure 4.2 Effect of Pd(II) concentration on complexation of trimeprazine at pH 4.7; (1) 50; (2) 200; (3) 400 ppm trimeprazine.

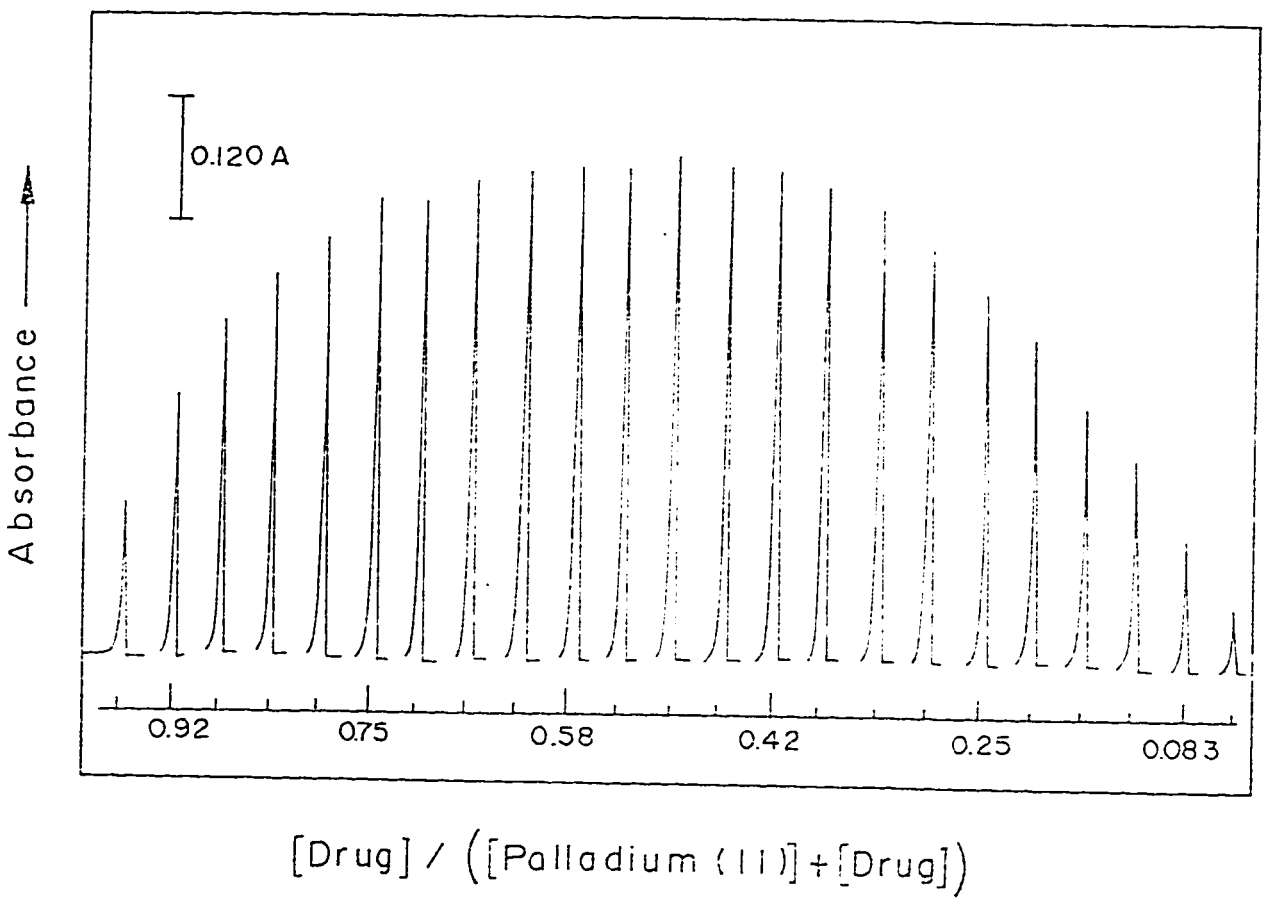


Figure 4 . 3 Job's plot; [Trimeprazine] = [Palladium(II)] = 8.25×10^{-4} M; Total volume is equivalent to 12.0 s (352.8 µl) and aspiration times were varied in 0.5 s steps.

Job's plot for this system was probed at pH 4.70, with typical plots as in figure 4.3. A plot of absorbance values versus molar fraction of palladium(II) using equimolar solutions of 8.24×10^{-4} M for palladium(II) and trimeprazine resulted in a stoichiometry of 1:1.

A calibration equation of the following form was obtained for the absorbance versus standard solutions of trimeprazine in the range of 50-400 ppm, with a correlation coefficient (r^2) of 0.993: $A = 9.0583 \times 10^{-2} + 1.5905 \times 10^{-3} C$; where A is absorbance and C is concentration of trimeprazine in ppm.

4.1.3 The Perphenazine System

The complex formation was fast with peak maximum at 560 nm and found to be stable for more than two days.

The effect of acidity on peak absorbance was typically represented by figure 4.4. It is quite obvious that optimization to maximum peak absorbance might lead to obtaining erroneous results due to the narrow limited stability range of pH; unless a suitable buffer is used for this system in particular in the range of 4 to 6 pH.

The effect of changing palladium concentration on peak absorbance resembles the above system, and as shown in figure 4.5, has no significant effect beyond 2.5×10^{-3} M at pH 4.85 for perphenazine in the range of 50 - 400 ppm.

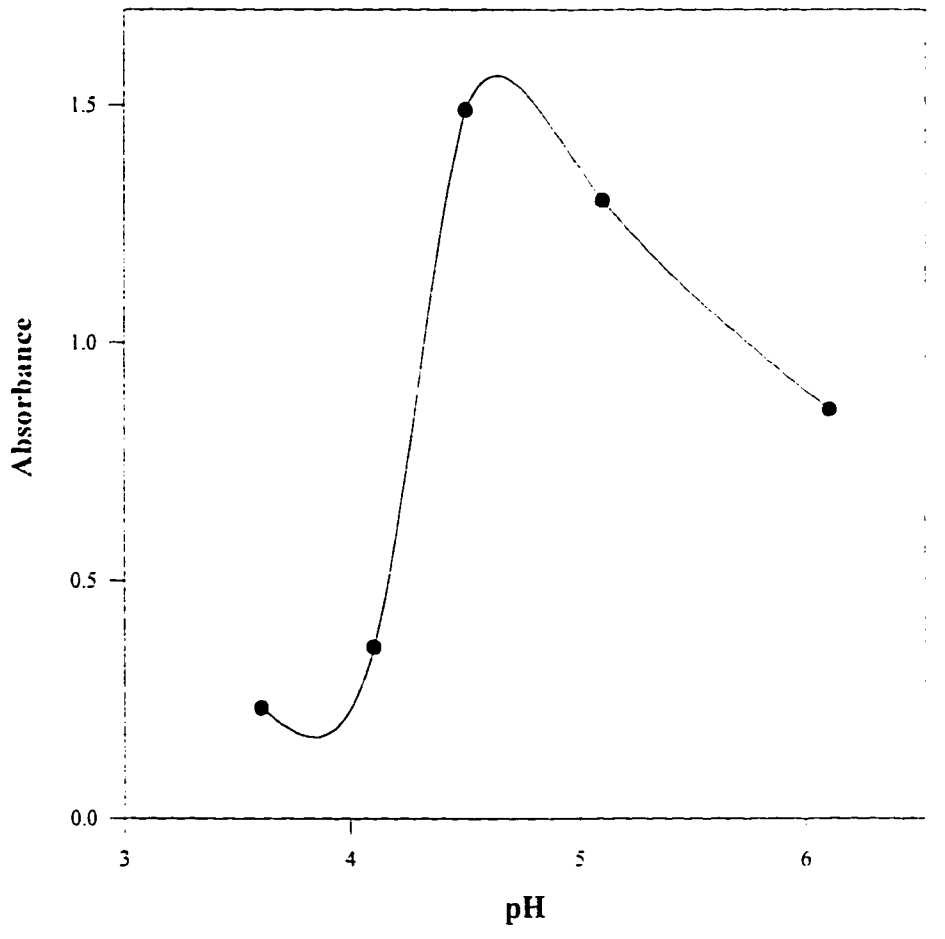


Figure 4.4 Effect of pH on complexation of 1.14×10^{-4} M Pd(II) with 300 ppm perphenazine.

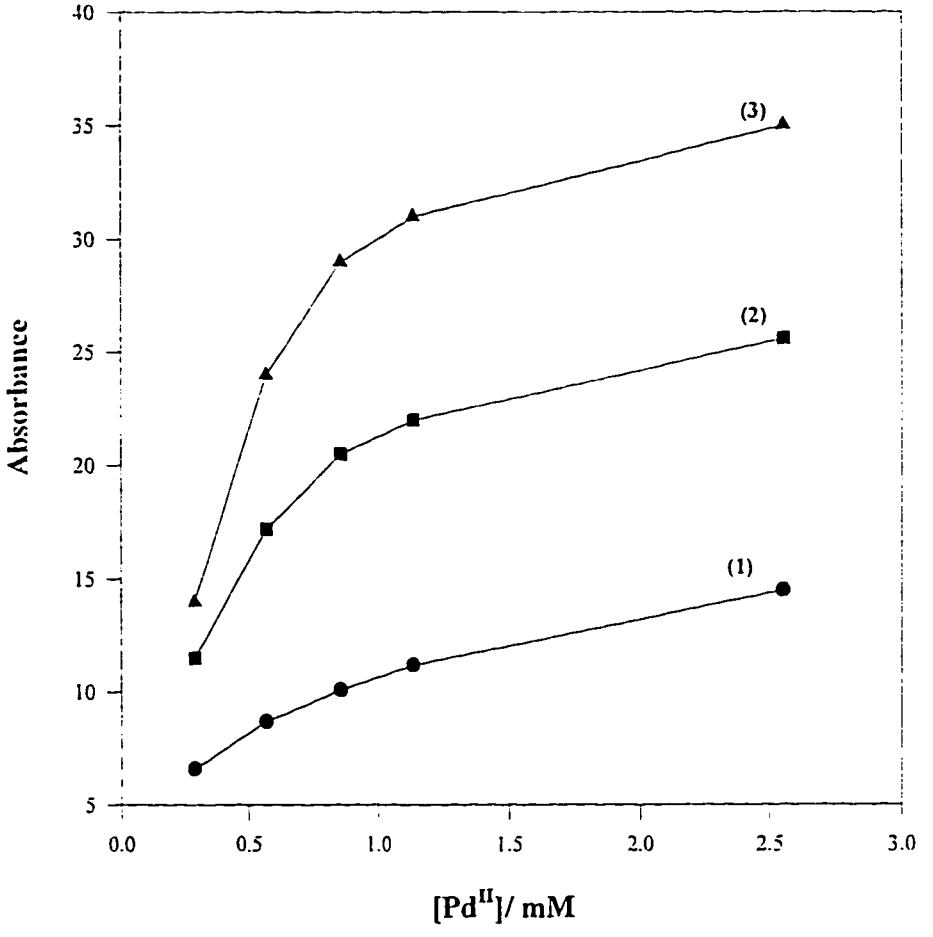


Figure 4.5 Effect of Pd(II) concentration on complexation of perphenazine at pH 4.85; (1) 50; (2) 200; (3) 400 ppm perphenazine.

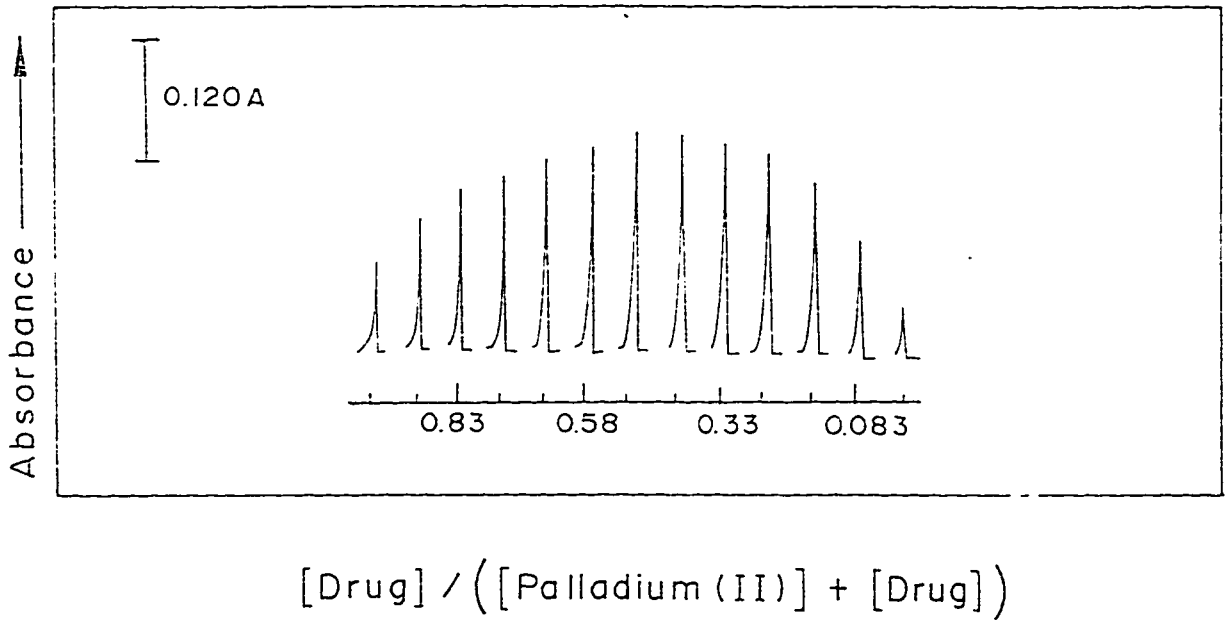


Figure 4 . 6 Job's plot; [perphenazine] = [Palladium(II)] = 5.70×10^{-4} M; Total volume is equivalent to 12.0 s (352.8 µl) and aspiration times were varied in 1.0 s steps.

Figure 4.6 is a typical Job's plot for this system conducted at pH 4.85 using equimolar solutions of 5.70×10^{-4} M for the drug and palladium. The stoichiometry was always 1:1.

The following calibration equation was obtained for the absorbance versus standard solutions of perphenazine in the range of 50 - 500 ppm with (r^2) of 0.992: $A = 8.5387 \times 10^{-2} + 1.5390 \times 10^{-3} C$

4.1.4 System Optimization

The instrument was operated at different flow rates but with fixed holding coil length of 100 cm and a reaction coil of 40 cm using 0.80 mm - i.d. PTFE tubings. Considering a maximum total volume of palladium and the drug of about 353 µl required to be aspirated into the holding coil in a reverse mode of the pump for 6 seconds for each reagent with a total measuring cycle of not much longer than one minute; a flow rate of 29.5 µl/s was adjusted by the pump for all cycles.

4.1.5 Analytical Appraisals

The method was applied to the determination of trimeprazine and perphenazine in synthetic samples containing some compounds usually added to pharmaceutical preparations as in table (4.1). From percentage recovery, starch, glucose, magnesium sulfate and citric acid exhibited no interference even when added at a level of ten times higher than the drug concentration. Sodium benzoate and ammonium acetate exhibited no interferences when added at levels up to twice the concentration of the drug.

Table 4.1 The results obtained by the SIA method for the analysis of 200 ppm of trimeprazine and perphenazine in presence of some selected compounds.

Compound added/ppm	Trimeprazine	Perphenazine
	% Recovery ± s d*	% Recovery ± s d*
Starch (2000)	100.14 ± 0.73	99.20 ± 0.52
Glucose (2000)	99.30 ± 0.86	101.50 ± 0.57
$MgSO_4$ (2000)	100.38 ± 0.70	99.80 ± 0.43
Citric acid (2000)	101.42 ± 0.91	99.5 ± 0.67
Na-benzoate (400)	101.77 ± 0.84	99.8 ± 0.83
Ammonium acetate (400)	99.92 ± 0.96	101.6 ± 0.98

* Standard deviation for 6 determinations.

The determination of trimeprazine and perphenazine of the same batch samples was carried out by the British Pharmacopoeia[107] and the United State Pharmacopoeia[108] methods. All these trials were deemed to have failed owing to high interferences of the above compounds and unsuitability of application to such low concentrations of the drugs using a titrimetric method. In this respect the present method is far superior to the official methods. It is superior to the other reported methods[6,7,93,95] with respect to stability of the reaction product and specificity. Additionally, the SI technique can easily be automated, thus requiring consumption of considerably less reagents and minimum labor.

4.2 **Determination of Ciprofloxacin and Norfloxacin**

Formation constants, or equilibrium constants for complexation reactions, is an effective measure of the affinity of a ligand to a metal ion in solution and is used to provide valuable information essential to many other fields e.g. medicine, electrochemistry, geochemistry, and pollution.[109,110] The advent of computers has enabled advanced mathematical treatment of huge equilibrium data to be carried out and also encouraged amateurish chemists to determine the formation constants by making use of advanced computerized numerical methods rather than just studying the position of equilibria they encounter during the course of their investigation of a chemical reaction. [109].

The aim of this work is to demonstrate the versatility of SI technique in conjunction with the numerical methods for the determination of stoichiometries and stability constants for the complexation of two members of the flouroquinolones family of drugs, the ciprofloxacin and the norfloxacin. Two FI methods based on the complexation reaction of iron(III) with ciprofloxacin and norfloxacin have been reported recently.[88,92] There-in, no detailed information with respect to the stability nor stoichiometry studies have been carried out on line by the FI micro-technique. The present work demonstrates the advantages of SI over FI technique thus showing the inevitability of the utilization of the SI-technique for such investigations. A method for the determination of ciprofloxacin and norfloxacin, based on their complexation with iron(III) in sulfuric acid medium, is also proposed. A chemometric optimization approach is employed to select the best experimental conditions for the assay of these two drugs.

4.2.1 Determination of Stoichiometry

4.2.1.1 General Consideration

In sulfuric acid concentration range 5.0×10^{-3} - 0.60 M, a deep yellow color is formed instantaneously when iron(III) solution is added to ciprofloxacin and norfloxacin solutions, indicative of complex formation. The spectrum of the complex shows an absorption band at 350 nm for

ciprofloxacin and 370 nm for norfloxacin with a shoulder that extends in the visible region. The shoulder, which is responsible for the yellow color of the complex, shows a maximum absorbance at 447 nm for ciprofloxacin and at 430 nm for norfloxacin. At these wavelengths the two drugs and iron(III) show negligible absorbance. It has also been observed that the intensity of the colored complex increases with a decrease in the concentration of sulfuric acid which may be due to the dissociation of the complex at high acid concentration.

4.2.1.2 Job's Plots

In the Job's method of continuous variation, different aliquots of equimolar solutions of iron(III) and of drug were mixed to give solutions of identical total concentration (iron(III) + drug) but different mole fractions. Therefore, a total volume of aspiration of 162.0 µl was maintained constant by adjusting the aspiration times. The volume of each reagent was varied between 14.8 µl (0.5 s) and 147.5 µl (5.5 s).

The Job's plot of continuous variation was used to determine the stoichiometry of the complexation of ciprofloxacin and norfloxacin with iron (III) in sulfuric acid media. Figure 4.7 is a typical Job's plot generated by using equimolar solutions of iron(III) and ciprofloxacin of 1.00×10^{-3} M, whereas figure 4.8 shows a Job's plot for equimolar solutions of iron(III) and norfloxacin of 2.00×10^{-3} M. Both plots were produced using 5.00×10^{-3} M sulfuric acid and an ionic strength of 0.20 M. It is clear that the curves A = $f(X_{drug})$ exhibit a maximum for the mole fractions close to 0.66, indicating that the ratio of iron(III) : drug in the complex is 1:2. Similar

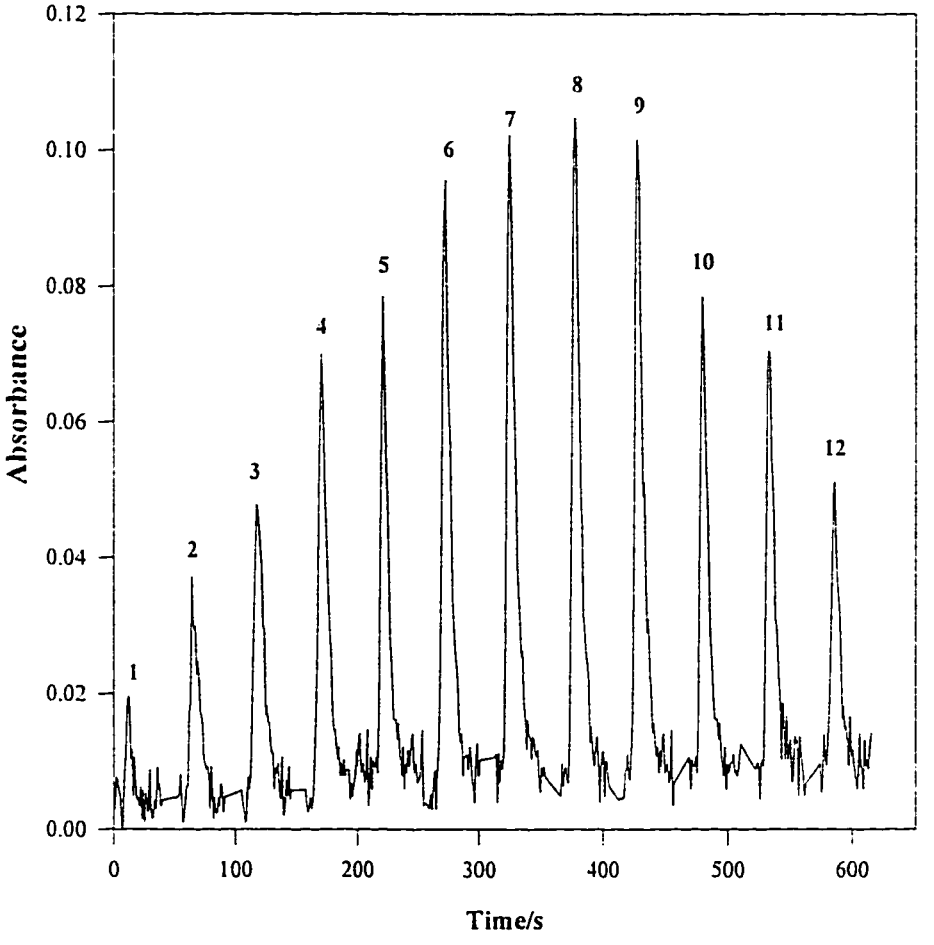


Figure 4.7 Siagram representing Job's plot for norfloxacin system; [iron(III)] = [ciprofloxacin] = 1.00×10^{-3} M; $[H_2SO_4] = 5.00 \times 10^{-3}$; ionic strength = 0.20 M; Total aspiration volume is equivalent to 162 µl and aspiration volumes were varied between 14.5 µl and 147.5 µl; mole fractions of the drug were (1) 0.0; (2) 0.1; (3) 0.2; (4) 0.3; (5) 0.4; (6) 0.5; (7) 0.6; (8) 0.7; (9) 0.8; (10) 0.9; (11) 0.95; (12) 0.97.

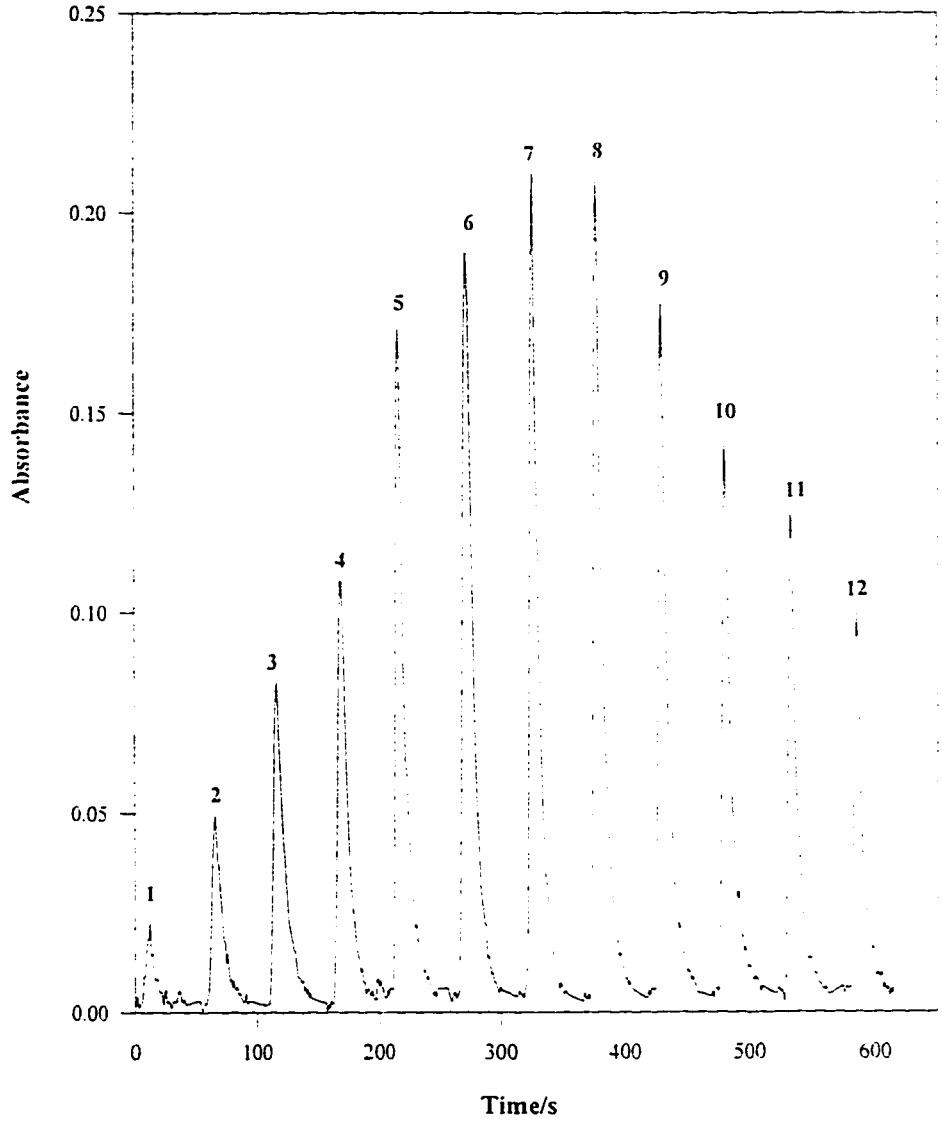


Figure 4.8 Siagram representing Job's plot for norfloxacin system; [iron(III)] = [norfloxacin] = 2.00 $\times 10^{-3}$ M; $[H_2SO_4]$ = 5.00 $\times 10^{-3}$; ionic strength = 0.20 M; Total aspiration volume is equivalent to 162 µl and aspiration volumes were varied between 14.5 µl and 147.5 µl; mole fractions of the drug were (1) 0.0; (2) 0.1; (3) 0.2; (4) 0.3; (5) 0.4; (6) 0.5; (7) 0.6; (8) 0.7; (9) 0.8; (10) 0.9; (11) 0.95; (12) 0.97.

plots made at sulfuric acid concentrations greater than 0.025 M, gave different results, and the 1:1 iron(III):fluoroquinolone complex was found to be the dominant species. These findings are in agreement with the results published by two independent groups, where 1:1 complex was obtained by one group[88] when iron(III) was complexed with ciprofloxacin at acidities higher than 0.025M, and 1:2 complexes were obtained for the complexation of norfloxacin by the other group[111] when 5.00×10^{-3} M sulfuric acid was used. It can also be observed that only ten minutes at most are needed to generate the Job's plot using SIA and less than 10 ml are enough to repeat the procedure for, at least, five times.

4.2.1.3 Molar Ratio Plots

In the molar ratio method ideally two straight lines are obtained when the absorbance is plotted versus the iron(III) to drug ratio, and the point of intersection of these two lines corresponds to the stoichiometric ratio upon interpolation to the mole ratio axis. Therefore the total volume of the ligand was maintained constant by aspirating 147.5 µl into the holding coil by flow reversal, whereas iron(III) solution was varied between 14.8 µl (0.5s) and 192.0 µl (6.5 s). The ionic strength of all solutions was adjusted to 0.20 M with ammonium sulfate and the sulfuric acid concentration was 5.00×10^{-3} M. Figure 4.9 shows a typical molar ratio plot for the complexation of iron(III) with ciprofloxacin, with peaks 1 to 11 produced by varying the molar ratio of iron(III) to drug from 0.1 to 1.1 in 0.1 steps. On the other

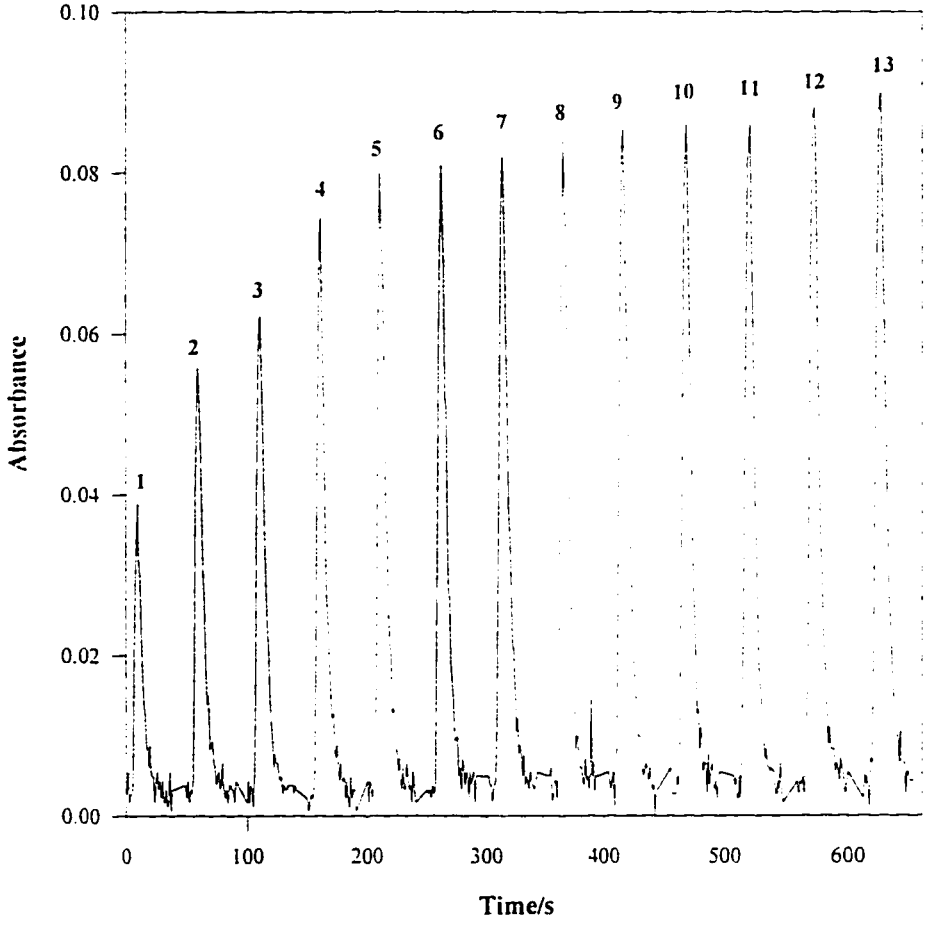


Figure 4.9 Siagram representing molar ratio plots for ciprofloxacin system; [iron(III)] = [ciprofloxacin] = 1.00×10^{-3} M; $[H_2SO_4] = 5.00 \times 10^{-3}$; ionic strength = 0.20 M; Total aspiration volume of the drug is equivalent to 147.5 µl and aspiration volumes of iron(III) were varied between 14.5 µl (Peak 1)and 192.0 µl (peak 13)

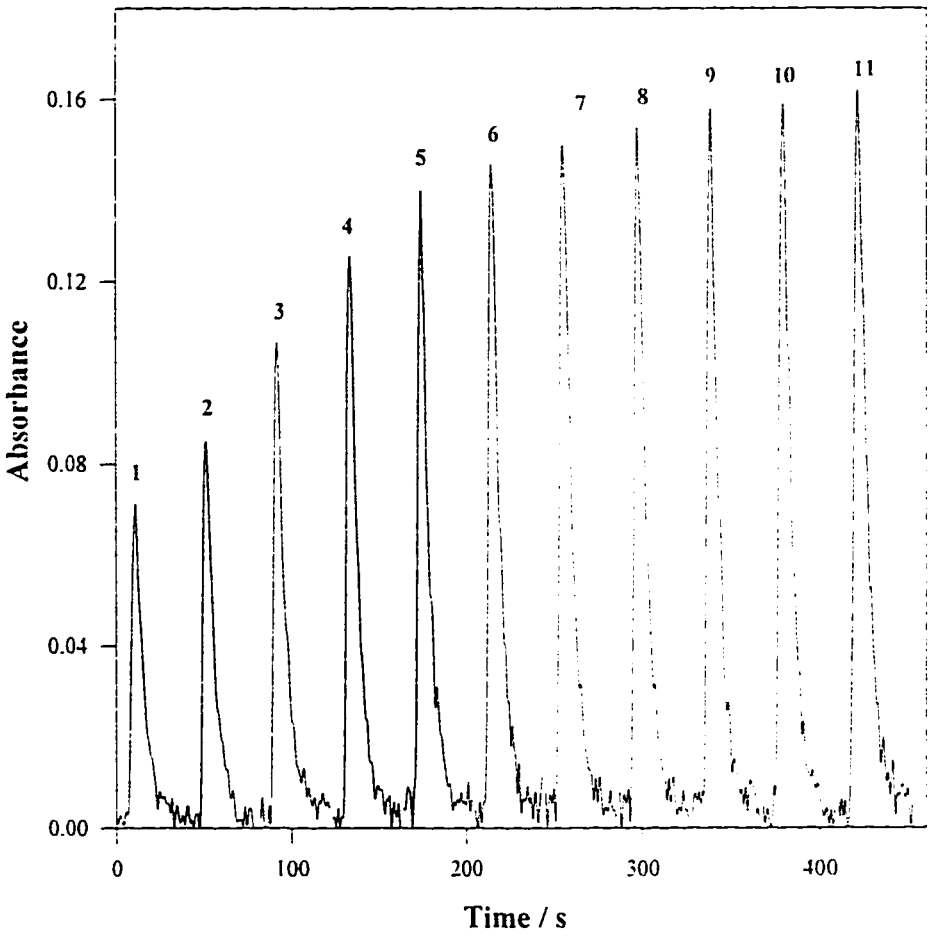


Figure 4.10 Siagram representing molar ratio plots for ciprofloxacin system; [iron(III)] = [ciprofloxacin] = 1.00×10^{-3} M; $[H_2SO_4] = 5.00 \times 10^{-3}$; ionic strength = 0.20 M; Total aspiration volume of the drug is equivalent to 147.5 µl and aspiration volumes of iron(III) were varied between 14.5 µl (Peak 1)and 162.0 µl (peak 11)

hand figure 4.10 represents a molar ratio method for the complexation of norfloxacin with iron(III), here the molar ratio of the iron(III) to the drug was varied from 0.1 (peak 1) to 1.3 (peak 13) in 0.1 steps. These plots were produced using equimolar solutions of iron(III) and the corresponding drug. The ionic strength was adjusted at 0.020M and sulfuric acid was at 5.00 x 10^{-3} M. It is obvious from these plots that the point of intersection of the two lines is close to 0.5 when extrapolation is made to the molar ratio axis, indicating a 1:2 ratio for iron(III) : drug.

It is likely that incomplete mixing of the reagents with the drug might take place due to dispersion and zone penetration, however, nesting the solutions around the valve followed by getting rid of the excess together with the automated aspiration with the exact computer control of timing would certainly minimize this problem. If any is still existing, it would lead to a constant error for both the drug and the reagent concentrations, that would pose no problem in the data obtained and in calculating the mole ratio. On the other hand the correction factors which can be introduced from the zone penetration and the dispersion were found to have a very minor effect on the data obtained.

It is well known that iron(III) has its greatest affinity for ligands that coordinate by oxygen.(112) It has also been reported (113,114) that fluoroquinolones are present in different forms in aqueous media, including neutral molecules, zwetterions as well as monoprotonated and diprotonated species depending on the pH of the media. For instance, at low acid concentrations the neutral species together with the monoprotonated species

are the predominant forms, whereas at highly acidic solutions the proportion of the diprotonated form increases greatly. Therefore it can be suggested that iron(III) complexes ciprofloxacin and norfloxacin by binding to the carboxylate group and to the adjacent keto-group in the 3 and 4 positions respectively, thus forming a six membered ring, as shown previously.(88,93)

4.2.2 Formation Constants

The formation constant and the composition of the complexation of iron(III) with ciprofloxacin and norfloxacin were also investigated by numerical methods. The Jobcon program(49) was used to analyze the continuous variation data whereas MRLET program was utilized to treat the molar ratio data. (50) Both programs were modified by us and were rewritten in C-language and applied on a PC/AT computer. The calculations are based on fitting a function $f(x,\beta)$ to a set of experimental data, using a least square method. Unknown parameters are estimated by minimizing U, the sum of squares of residuals defined by equation 1.

$$U = \sum_{i=1}^{n} (A_{expi} - A_{calci})^2 \qquad 4.1$$

where n represents the number of experimental points, A_{exp} the experimental absorbance, and $A_{calc} = f(x,\beta)$, the absorbance calculated by the program from the formation constants and the stoichiometric ratios. Therefore, various equilibrium models could be fitted to the experimental data iteratively by varying the values of formation constants and those of the stoichiometric ratios.

Table 4.2 Computer output from Jobcon program for the ciprofloxacin system[#].

m : n*	$\text{Log } K_f^{'}$	RE%
1 : 1	3.171	48.05
1 : 2	7.673	12.00
1 : 3	11.728	21.94
1 : 4	16.860	28.00
2 : 1	6.715	80.13
3 : 1	10.270	96.79
4 : 1	13.636	80.78

[iron(III)] = [ciprofloxacin] = 2.00×10^{-3}

* metal : ligand mole ratio

Table 4.3 Computer output from Jobcon program for norfloxacin system

m : n*	Log $K_f^{'}$	RE%
1 : 1	3.167	54.41
1 : 2	7.673	5.09
1 : 3	11.728	300.0
1 : 4	16.860	21.90
2 : 1	6.715	81.90
3 : 1	10.270	83.68
4 : 1	13.636	82.33

[iron(III)] = [norfloxacin] = 2.00×10^{-3}

* metal : ligand mole ratio

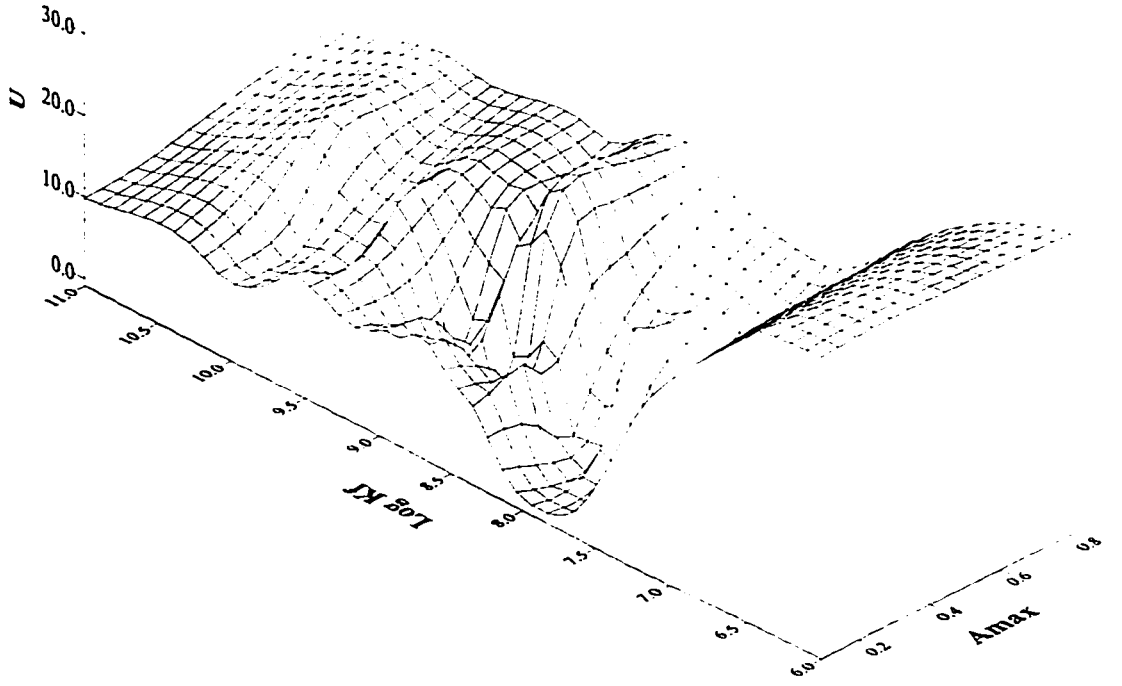


Figure 4.11 Surface plot of U as a function of $\log K_f^{'}$ and the maximum absorbance from the Merlet program for norfloxacin system.

Table 4.2 shows part of a computer output for the iron(III)-ciprofloxacin system using Jobcon program for the calculation of the formation constants from continuous variation data. It is clear from this table that the 1 : 2 metal : ligand ratio is the dominant one with a relative error of 11.99% and resulting in a value of logarithm of formation constant (log K_f) of 7.673. Other metal : ligand ratios (m:n) deemed to be improbable with high relative errors, especially those where m : n > 2. Table (4.3) shows another part of Jobcon computer program output, where the 1 : 2 metal : drug ratio is confirmed to be the correct mole ratio for iron(III)-norfloxacin system. The value obtained here for the logarithm of the formation constant (log K_f) is 7.747 with a relative error of 5.08%.

Figure 4.11 is a surface plot of the minimization function (U), given by equation 4.1, as a function of log K_f and the maximum absorbance (A_{max}) for the iron(III)-norfloxacin system, obtained from the Merlet program. A_{max} corresponds to the absorbance of the complex if there was no dissociation, and is equivalent to the absorbance of the intersecting tangents of the molar ratio plot. It is clear from figure 4.11 that a global minimum is almost obtained for log K_f values close to 7.850, indicating that this is the optimum value of log K_f. Similar plots were generated for the iron(III)-ciprofloxacin system, with an optimum log K_f value of 7.774. The mean values of log K_f obtained by these numerical methods for ciprofloxacin and norfloxacin complexes with iron(III) in 5.00 x 10^{-3} M sulfuric acid and 0.20 M ionic strength, were 7.756 ± 0.121 and 7.839 ± 0.056 respectively.

The value of log K_f reported earlier for the norfloxacin was 8.60.[111] It is apparent from these results, that the value of the formation constant for the 1:2 iron(III) - norfloxacin is close to the value reported earlier.[111] It was also observed that the stoichiometry and the value of formation constant are independent of the total concentration of the metal ion and the ligand.

4.2.3 Optimization

The variables included in the optimization were, aspiration volume and concentration of iron(III), which were found to be of great influence on the peak absorbance. Preliminary investigations have revealed that other performance criteria such as the precision are of acceptable levels. Three levels of the aspiration volume were included, these are 29.5 µl, 88.5 µl and 147.5 µl, whereas five levels of iron(III) were selected in the range 2.0 $x10^{-3}$ M - 10.0 $x10^{-3}$ M. Iron(III) concentration was varied at each level of aspiration volume until no further improvement in the peak absorbance was observed, then five levels of iron(III) were selected to construct a factorial design. The factorial design was then repeated by nesting the selected iron(III) solutions around the selector valve in a randomized manner in an attempt to minimize any possible bias. In all of these experiments 88 µl of 200 ppm of the appropriate drug was aspirated, and all experiments for each drug were run within one hour.

The factorial design experimental data for ciprofloxacin and norfloxacin systems are given in tables 4.4 and 4.5 respectively. In order to

Table 4.4 Factorial design for optimization of iron(III)concentration and aspiration volume for ciprofloxacin system.

[iron(III)]		Aspiration volume		peak height/mm
mM	Coded level	µl	coded level	
2.0	-1	147.5	1	50
4.0	-0.5	147.5	1	58
6.0	0	147.5	1	62
8.0	0.5	147.5	1	65
10.0	1	147.5	1	64
2.0	-1	88.5	0	47
4.0	-0.5	88.5	0	57
6.0	0	88.5	0	62
8.0	0.5	88.5	0	65
10.0	1	88.5	0	65
2.0	-1	29.5	-1	36
4.0	-0.5	29.5	-1	48
6.0	0	29.5	-1	55
8.0	0.5	29.5	-1	59
10.0	1	29.5	-1	56

Table 4.5 Factorial design for optimization of iron(III) concentration and aspiration volume for norfloxacin system.

[iron(III)]		Aspiration volume		peak height/mm
mM	coded level	μl	coded level	
1.0	-1	147.5	1	46
2.0	-0.5	147.5	1	58
4.0	0	147.5	1	68
6.0	0.5	147.5	1	73
8.0	1	147.5	1	76
1.0	-1	88.5	0	34
2.0	-0.5	88.5	0	50
4.0	0	88.5	0	61
6.0	0.5	88.5	0	68
8.0	1	88.5	0	72
1.0	-1	29.5	-1	17
2.0	-0.5	29.5	-1	32
4.0	0	29.5	-1	42
6.0	0.5	29.5	-1	51
8.0	1	29.5	-1	56

determine the optimum experimental conditions for the assay of these drugs, based on their complexation with iron(III) in sulfuric acid media, an ordinary least square method[28] was used together with the exhaustive search for the best regression model (all model search) to find a model which correlates peak absorbance with the concentration and aspiration volume of iron(III). Before undertaking any statistical analysis the factors selected were scaled within the range ±1 to simplify the interpretation of the regression coefficients when the relative importance of factors is compared.

The all-model search was utilized to investigate all possible models with 1,2,..., k parameters. Quadratic, cubic and two- factor interaction were included to obtain the best model. The test statistic used here is an F- ratio of the form[115]

$$F = \frac{(SSE_R - SSE_C)/r}{SSE_C/(N-P)} \qquad 4.2$$

Where SSE_R and SSE_C are the residual sum of squares associated with the reduced model and the complete model i.e. models with i -1 and i parameters, respectively, r is the difference in the number of parameters in the reduced and complete models, p is the number of parameters in the complete model and N is the number of experiments. The best model is that is significant to the F-test described by equation 4.2 and characterized by the highest coefficient of multiple regression (R^2). Table (4.6) shows

Table 4.6 Comparison between the best models for the optimization of norfloxacin system.

No. of parameters in the model	SSE#	DF*(N-p)	R^2	$F^{\otimes}_{calculated}$	$F^{\varnothing}_{theoretical}$
3	312.7	12	0.917	-----	-----
4	219.1	11	0.936	4.70	6.72
5	115.4	10	0.963	8.99	6.94
6	73.3	9	0.974	5.16	7.21
7	54.1	8	0.978	2.84	7.57

sum of squares of errors.

* degrees of freedom.

⊗ F-ratio calculated by equation 4.2.

∅ theoretical F-value at 97.5% significant level.

the comparison between the best regression models obtained for norfloxacin system. It can be revealed from this table that the five parameter model (one intercept and four independent variables) is the best model that can be used to describe the response surface of the norfloxacin system. Comparison between the best five parameter model and the best four parameter model by the F-test given above shows that the test is significant at the 97.5% level with a value of 8.99. Table (4.7) shows the comparison between the best models obtained for ciprofloxacin system. It is clear that the best three, four and five parameter models are all significant to the F-test of equation 4.2. However, the five parameter model is considered superior to the other models because it is characterized by the best correlation of multiple variation. Therefore, the five parameter model is selected as the best model for the ciprofloxacin system. The final forms of these five parameter models obtained for ciprofloxacin and norfloxacin systems are given below respectively:

$$PH = 62.63 + 8.67\,I + 4.40\,V - 6.86\,I^2 - 4.00V^2 \quad (R^2 = 0.973) \qquad 4.3$$

$$PH = 60.94 + 17.87\,I + 12.91\,V - 6.29\,I^2 - 5.30V^2 \quad (R^2 = 0.964) \qquad 4.4$$

where PH is the peak height in mm, I is the iron(III) concentration and V is the aspiration volume.

The predictive ability of the selected model was evaluated by the leave-one-out cross validation method. In this method the predicted responses are calculated by using the model adopted and by excluding the

Table 4.7 Comparison between the best models for the optimization of ciprofloxacin system.

No. of parameters in the model	SSE#	DF*(N-p)	R^2	$F^{\otimes}_{calculated}$	$F^{\varnothing}_{theoretical}$
2	396.4	13	0.587	-----	-----
3	202.8	12	0.753	11.5	9.33
4	79.4	11	0.917	17.1	9.65
5	26.0	10	0.972	20.5	10.04
6	13.2	9	0.986	8.70	10.56

sum of squares of errors.

* degrees of freedom.

⊗F-ratio calculated by equation 4.2.

∅theoretical F-value at 97.5% significant level.

particular data point for which the prediction is to be made. Figures 4.12 and 4.13 show the leave-one-out- cross validated responses plotted as a function of the experimentally observed responses for ciprofloxacin and norfloxacin systems respectively. The coefficient of multiple determination (R^2) obtained from these figures are 0.972 and 0.967 for the ciprofloxacin and norfloxacin systems respectively, indicating that excellent fit for the experimental responses can be obtained by these models. Also it can be concluded that the predictive abilities for these models are excellent suggesting that they can be safely used for optimization purposes. Determination of the optimum experimental conditions for the assay of the two drugs was then executed by a modified simplex algorithm as a search method and the regression models obtained above as objective functions. The optimum experimental conditions thus obtained by this strategy were 147.5 µl for the aspiration volumes for both systems whereas 1.00×10^{-2} M and 8.0×10^{-3} M values were obtained for iron(III) concentration for ciprofloxacin and norfloxacin systems respectively. Figure 4.14 shows a surface plot as a function of iron(III) and aspiration volume for ciprofloxacin system. It is apparent from this figure that higher levels of both iron(III) and aspiration volumes are favorable for the assay of this drug as the peak absorbance reaches a plateau region.

4.2.4 Analytical Appraisal

Series of standard solutions were run for the two drugs as typically shown in figures 4.15 and 4.16 for ciprofloxacin and norfloxacin respectively. A weighted regression line was plotted for the absorbance

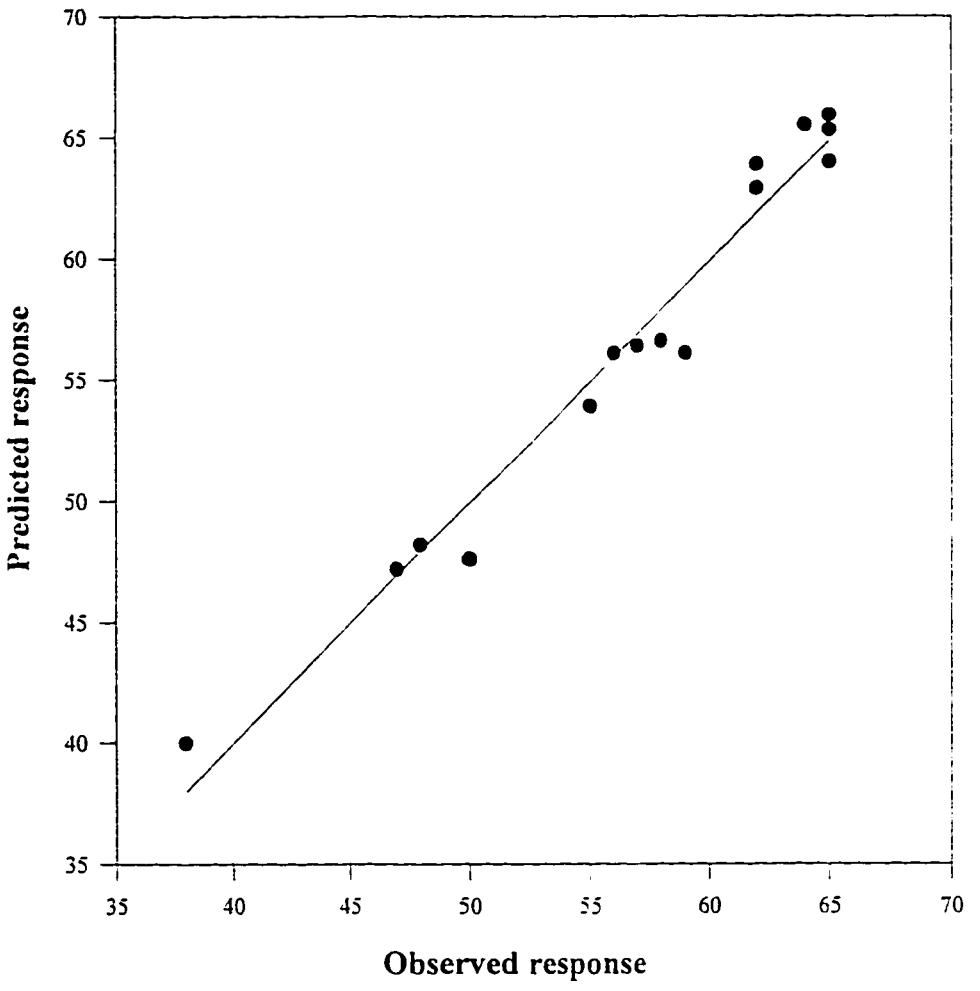


Figure 4.12 Predicted response obtained by the leave-one-out method vs. the experimentally observed response for the ciprofloxacin system.

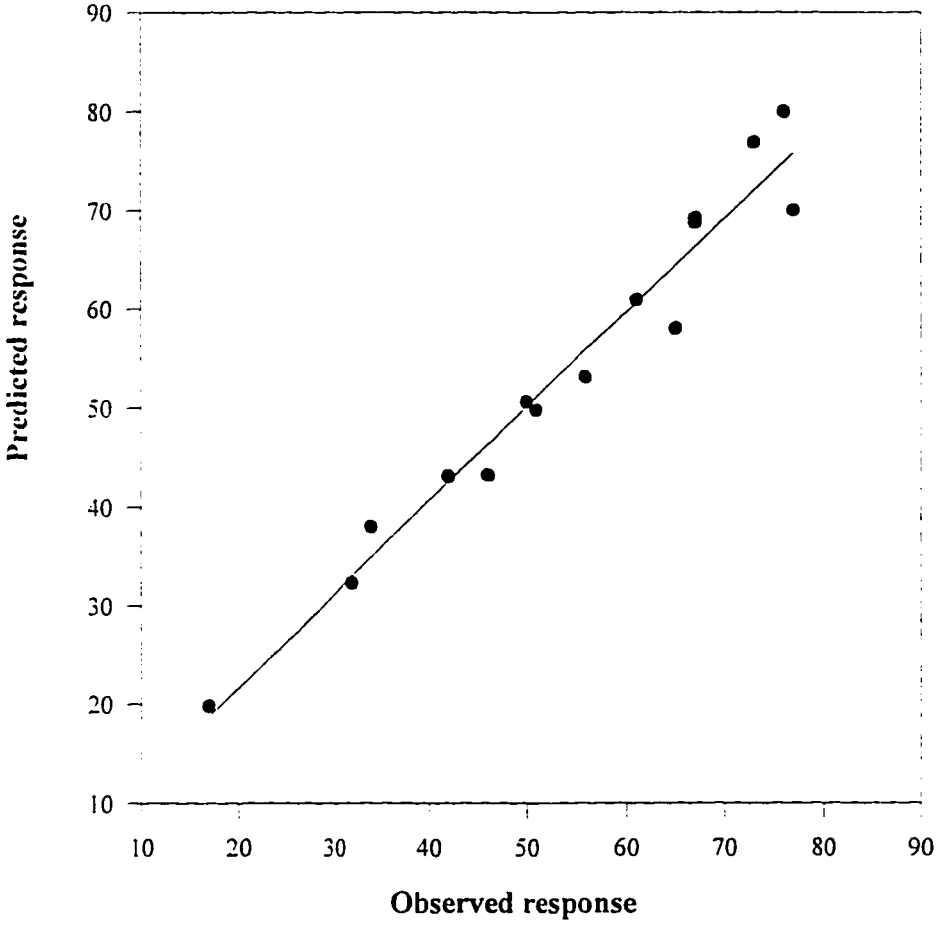


Figure 4.13 Predicted response obtained by the leave-one-out method vs. the experimentally observed response for the norfloxacin system.

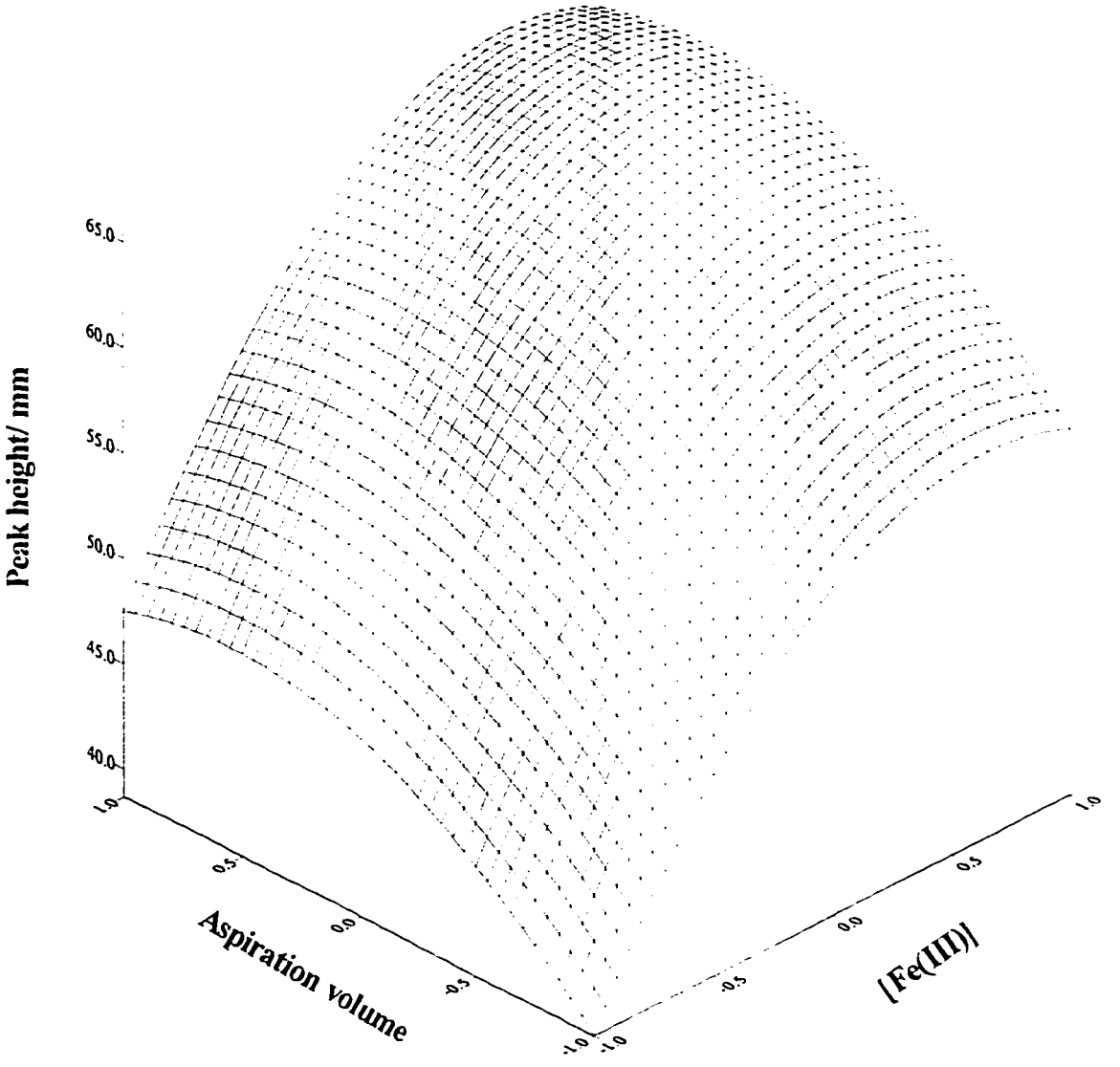


Figure 4.14 Response surface plot of peak absorbance as a function of iron(III) concentration and aspiration volume.

versus concentration by giving minimum weighting for the lower and upper concentrations and full weighting for the others. The linear range was found to be between 50 and 500 ppm for ciprofloxacin and between 50 and 400 ppm for norfloxacin with the following calibration equations:

$$A = 0.0280 + 4.592x\ 10^{-4}\ C\ \ (R^2 = 0.995) \qquad 4.5$$

$$A = 0.0115 + 6.451x\ 10^{-4}\ C\ (R^2 = 0.999) \qquad 4.6$$

where A is the peak absorbance and C is the concentration in ppm.

4.2.5 Application

The proposed method was applied to the assay of some proprietary drugs containing ciprofloxacin and norfloxacin and these are listed in table (4.8). CibroBay tablets and CiproBay infusion(Bayer) with five runs represented by the groups of peaks 7 and 8 respectively in figure 4.15 and Noroxin tablets (Merk, Sharp and Dohme) represented by the group of peaks 7 in figure 4.16. The method was statistically compared to the previous FIA methods[88,93] and the results obtained (Table 2) indicate that a similar degree of accuracy can be obtained by the SIA and the FIA methods . In the FIA-methods both drugs were injected into a continuous stream of iron(III) solution to form a brown-red complex which is monitored at 447 nm and 430 nm for ciprofloxacin and norfloxacin respectively. It can also be suggested from these results that the proposed method is suitable for the determination of these two drugs in

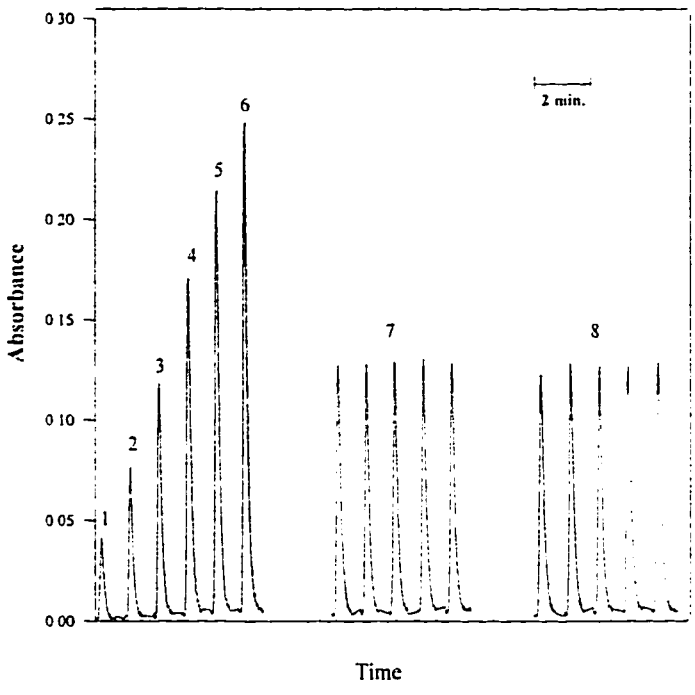


Figure 4.15 Typical SI-results for ciprofloxacin standard solutions of: (1) 50; (2) 100; (3) 200; (4) 300; (5) 400; (6) 500 ppm; (7) five runs of 215 ppm CiproBay tablets; (8) 210 ppm of CiproBay infusion.

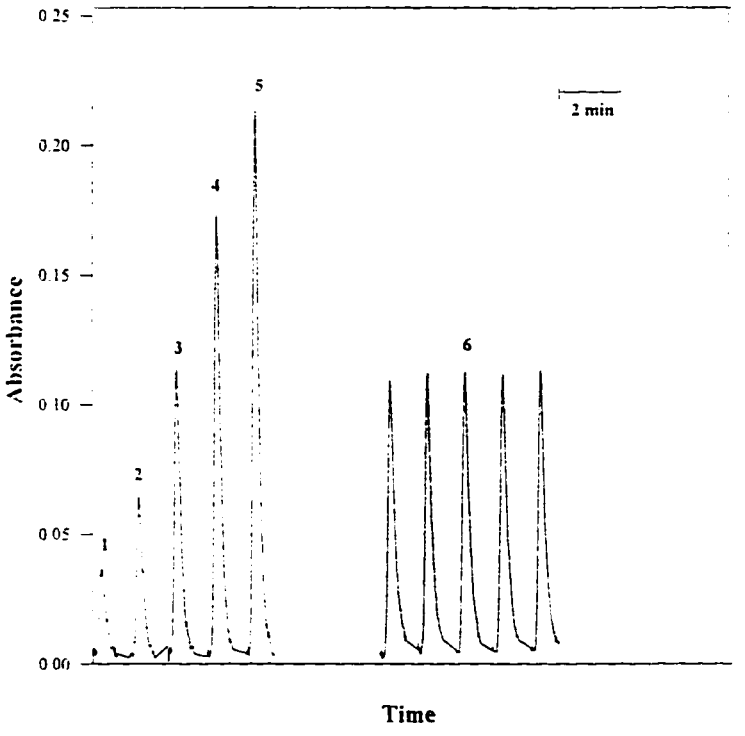


Figure 4.16 Typical SI-results for norfloxacin standard solutions of: (1) 50; (2) 100; (3) 200; (4) 300; (5) 400; (6) five runs of 200 ppm Noroxin tablets.

Table 4.8 Results obtained by SIA proposed method and FIA-method[88,93] for analysis of ciprofloxacin and norfloxacin in proprietary drugs.

Proprietary drug	**Supplier**	**Active material**	**Mean recovery ± SD(%)***		**t#**
			SI-method	FI-method	
CiproBay tablets	Bayer	Ciprofloxacin 250 mg	100.8 ± 0.94	101.8 ± 0.96	2.38
CiproBay infusion	Bayer	Ciprofloxacin 2000 ppm	99.4 ± 1.0	100.3 ± 0.68	2.01
Noroxin tablets	Merk, Sharp and Dhome	norfloxacin, 400 mg	99.8 ± 0.67	100.7 ± 0.55	2.31

* n = 5

calculated t-test values [theoretical value = 2.78 (p = 0.05)]

pharmaceutical products without fearing any interferences from compounds usually added to such formulations. The SIA-method is superior to the FIA-methods with respect to consumption of considerably less reagents. Although the later is characterized by a high sampling frequency the former is easier to operate and optimize since only a single pump and a single valve are used. Also the easiness of the use of the automated SI-system rendered the generation of a plenty of data in a short period of time a simple task.

4.3 Determination of Oxprenolol

Oxprenolol is 1-[(methylethyl)amino]-3- [2-(2-propenyloxy) phenoxy]-2- propanol. It is a non-selective b-blocker recognized as being an important drug and usually prescribed for the treatment of hypertension, angina pectoris, migraine and disfunctional labor.(116-118) Various methods for the assay of this drug have been reported in the literature.(119-121) A kinetic method based on oxidation of this drug by cerium(IV) in acidic media has also been described using the fixed time method. (37) The method was claimed to have a wide dynamic range, but surprisingly a very low sensitivity was observed.

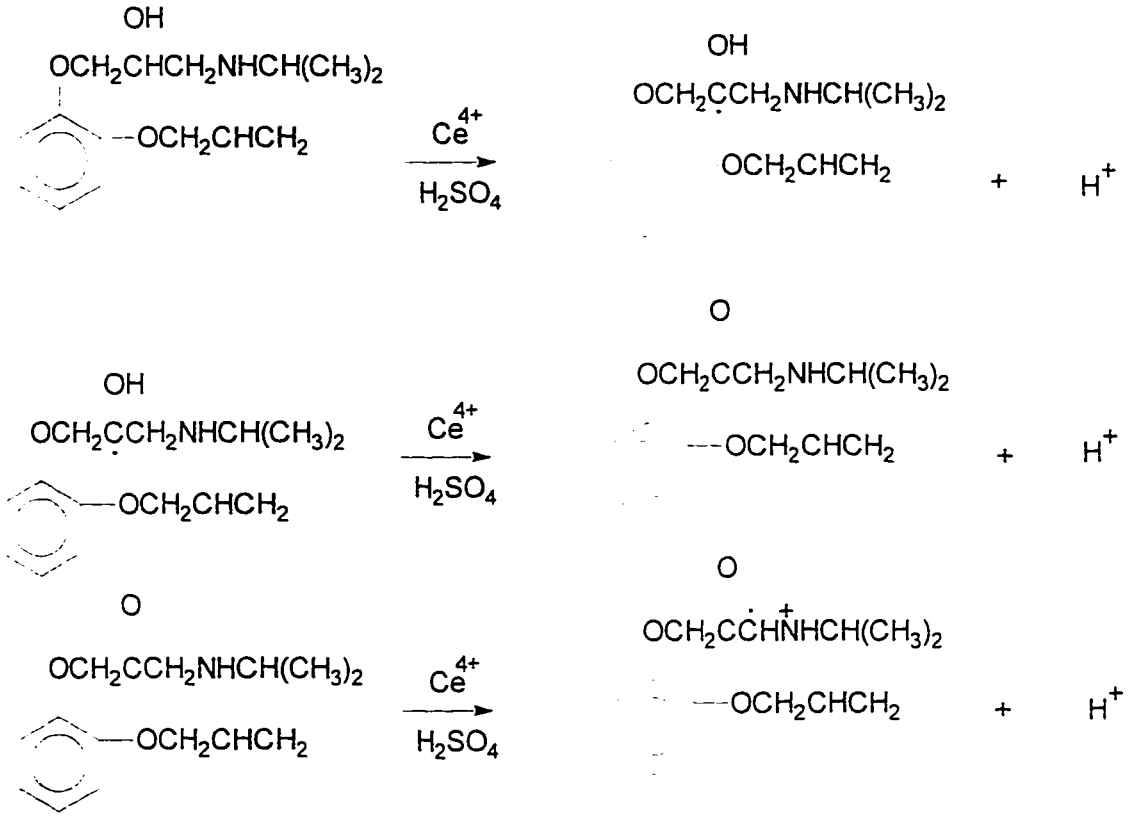
Orthogonal array designs are utilized to obtain the optimum conditions for the quantitative assay. These designs are also known as

Tagushi designs and were first introduced to analytical chemistry in 1989 by Oles and Yankovich[122] to optimize the operating conditions of gas chromatographic analysis. Orthogonality of a design is said to be obtained when the effect for a given factor (A) is calculated the influence of the other factors investigated cancel out. In other words, when the effect for factor (A) is merely dependent on the difference between the average of the responses for this factor at its levels. Orthogonal array designs were believed to incorporate the advantages of simplex method and factorial designs. [123]

This work involves the development of a simple method for the assay of oxprenolol utilizing SI-technique. The optimization of the operating conditions is probed by the orthogonal array design. The method is based on the oxidation of oxprenolol with cerium(IV) in sulfuric acid media.

4.3.1 The Chemical System

The oxidation of oxprenolol with cerium(IV) in sulfuric acid media generated a red-brown color that gave a maximum absorbance at 480 nm. The intensity of the colored product increases with an increase in acid concentration. It was observed that at acid concentration below 0.06 M the formation of the oxidation product is relatively slow but it was found to be accompanied by a precipitate formation. At higher acidtities the product was formed instantaneously, persists for few seconds and then starts to diminish.



Scheme 4.1 Reaction mechanism for the oxidation of oxprenolol with Ce(IV).

Oxprenolol side chain in the 2-position (N-alkylethanolamine group) is oxidized in a first step to generate an intermediate radical which reacts further to generate a stable ketone in a second slow step. It has also been reported[124] that the N-alkyl ethanal side chain can also undergo oxidation to produce a highly unstable dication diradical which might further disproportionate in highly acidic media. These steps are depicted in scheme 4.1.

4.3.2 Optimization

The most important factors anticipated to influence the formation of the colored product, which is believed to be the radical species, included in the optimization are cerium(IV) concentration (factor A), sulfuric acid concentration (factor B) and the stop time of the composite reaction zone at the reaction coil (factor C). At first, a two-level orthogonal array design denoted by $OA_8(2^7)$ was embarked to screen the response surface and to check for the existence of interaction between factors. Eight experimental trials were pre-designed to the two-level design and responses are obtained. The design matrix of the two-level design together with the corresponding responses is given in table (4.9). Factor D appearing in the design was aimed to act as a dummy variable that would help in calculating the error variance. The ANOVA table including the percent contribution (PC) is given in table (4.10). The percent contribution is calculated as follows:

$$PC = \frac{SS'}{SS_{total}} \times 100 \qquad 4.7$$

Table 4.9 $OA_8(2^7)$ matrix with the response values; oxprenolol concentration was 100 ppm.

[Ce(IV)]	$[H_2SO_4]$	Time	Factor D	Peak height / mm
1	1	1	1	11.2
1	1	2	2	10.0
1	2	1	2	24.5
1	2	2	1	19.5
2	1	1	2	21.0
2	1	2	1	18.0
2	2	1	1	35.0
2	2	2	2	32.0

Factor	High level (2)	Low level(1)
[Ce(IV)], A	8.0 mM	2.0 mM
$[H_2SO_4]$, B	2.6 M	1.2 M
Time, C	60	0

Table 4.10 ANOVA table with percent contribution.

Source of variation	SS	DF	mean square	F-ratio	$SS'^{@}$	PC[@]
[Ce(IV)], A	203.01	1	203.01	136.6*	201.51	36.28
$[H_2SO_4]$, B	316.26	1	316.26	212.8*	314.77	56.67
Time, C	30.81	1	30.81	20.73#	29.3	5.27
AB	1.71	1	1.71	1.15	0.226	0.04
BC	0.66	1	0.66	---	---	---
Residual	2.97	2	1.49	---	9.63	1.73
Total	555.43	7	---	---	555.43	100

@ see text

theoretical F-value is 18.5 ($p < 0.05$)

* theoretical F-value is 98.5 ($p < 0.01$)

where SS' is the purified sum of squares and is given by SS-MS_{error}; where SS is the individual sum of squares and MS_{error} is the square error; SS_{total} is the total sum of squares. From table (4.10), it is obvious that all factors are significant at $p < 0.05$, whereas only factor A and factor B are significant at $p < 0.01$. It is clear that factor A and Factor B are more overwhelming than factor C, this is further confirmed by the per cent contribution (PC) where factor B is the most contributing factor to the response (56.67 %) then factor A (36.28%), and factor C is the least contributing factor to the response by only 5.27%. Also the ANOVA table (table 24.10) has revealed that all the interactions between factors are insignificant. The per cent contribution due to the error is quite low, indicating that no important factor or interaction have been neglected in this design.

It is observed from the two level design that the best response was obtained when factor A(cerium(IV)) and factor B (sulfuric acid) are at their higher level whereas factor C (time) at the low level. It was therefore decided to further investigate additional levels of these factors in an attempt to model the response surface and to obtain better operating conditions. A four level orthogonal array design denoted by $OA_{16}(4^5)$ was selected, where each factor was investigated at four different levels. The design matrix together with the responses obtained is given in table (4.11). The ANOVA including per cent contribution was calculated and is shown in table (4.12). The F-test results shown in this table as well as the per cent contribution (PC) values further confirm the findings obtained by the two level design. It is clear that factor C is the least contributing factor, also the per cent contribution of the error is low indicating that no important factor or

Table 4.11 $OA_{16}(4^5)$ matrix with the response values; oxprenolol concentration was 100 ppm.

[Ce(IV)]	$[H_2SO_4]$	Time	Factor D	Peak height / mm
1	1	1	1	9.8
1	2	2	2	16.6
1	3	3	3	19.4
1	4	4	4	22.1
2	1	2	4	13.2
2	2	1	3	25.0
2	3	4	2	25.7
2	4	3	1	24.5
3	1	3	2	16.7
3	2	4	1	22.8
3	3	1	4	33.6
3	4	2	3	32.0
4	1	4	3	19.9
4	2	3	4	29.0
4	3	2	1	33.7
4	4	1	2	37.5

	Levels			
Factor	1	2	3	4
[Ce(IV)], mM	2.0	4.0	6.0	8.0
$[H_2SO_4]$, M	2.0	2.5	3.0	3.5
Time, s	0	20	40	60

Table 4.12 ANOVA table with percent contribution for $OA_{16}(4^5)$ design.

Source of variation	SS	DF	mean square	F-ratio	$SS'^{@}$	$PC^{@}$
[Ce(IV)], A	377.35	3	125.79	72.0*	375.61	40.36
$[H_2SO_4]$, B	500.78	3	166.92	95.5*	499.04	53.63
Time, C	41.98	3	13.99	8.0#	40.13	4.31
Residual	10.48	6	1.74	---	15.82	1.70
Total	930.60	15	---	---	930.60	100

@ see text

theoretical F-value is 4.76 ($p < 0.05$)

* theoretical F-value is 9.78 ($p < 0.01$)

interaction have been neglected. Therefore it is reasonable to neglect all two factor interaction. The best response values were obtained at the highest levels of cerium(IV) and the highest level of the acid but at the lowest level of the time. Therefore the optimum operating conditions for the assay of oxprenolol are 8.0 mM cerium(IV) and 3.5 M sulfuric acid and the reaction mixture is pumped directly to the detector i.e. no stop period in the reaction coil is necessary.

4.3.3 Analytical Application

Using the above conditions the following calibration equation was obtained for the absorbance versus standard solutions of oxprenolol in the range of 50-400 ppm with (R^2) of 0.991: $A = 9.539 \times 10^{-2} + 1.6340 \times 10^{-3} C$; where A is the absorbance and C is the concentration in ppm.

The method was applied to the determination of oxprenolol in the proprietary drug Trisicor-160 mg oxprenolol (Ciba-Giegy) with a percent recovery of 99.45% and a relative standard deviation of 0.88%. The method is superior to other methods reported in that it is fast and being an SI-method low consumption of reagents and drug is another advantage worth mentioing.

4.4 Kinetics and Sequential Injection Analysis

The work presented here is a typical demonstration for the capacity and versatility of the sequential injection (SI) as a powerful technique which

goes beyond reaction rate measurements and superficial kinetic studies undertaken in flow injection.[1,19,38,98] For the first time, the (SI) technique is employed for a full kinetic investigation leading to a direct postulation of a reaction mechanism thus validating a comprehensible method of analysis. This opens a way to mechanistic scientists to use an easy computer-controlled instrument they have been lacking for decades to fill the gap and replace the time-consuming manual dilution procedures being carried out to date. The remarkable development of the sequential injection analysis (SIA) technique to the subject of this work is mainly due to the following advantages :

1. With (SIA) reaction rate measurements can easily be adjusted and monitored through the duration of a stopped-flow period.
2. The SIA allows flexible selection of the injected volume easily, by a computer-controlled piston stroke.
3. With SIA a valve could be assigned for a mixing chamber where reactants could be mixed, stored to generate a set of diluted standard solutions or arrested for kinetic measurements.
4. With SIA minimum zone penetration and sample distribution could be obtained by the flow reversal capability of the system and by the precise volume aspiration computer-controlled uptake.

4.5 Kinetic Determination of Bromazepam

Bromazepam abbreviated as (BRZ) through out the text is chemically (7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one). It is a

psycho tropic drug used as a sedative, formerly known as a tranquilizer and commonly used to reduce pathological anxiety, tension, agitation and depression. It alters the mental state by affecting the neurophysiological and biochemical activity of the functional units of the central nervous system (CNS). Several methods for bromazepam determination have been proposed and summarized in a recent publication[37].

In this work the complexation reaction of bromazepam with iron(II) in hydrochloric acid media was fully investigated. The kinetics of the reaction, reaction order with respect to each reactant, the kinetic energy were all determined, and the mechanism was postulated. Finally a kinetic method for the determination of bromazepam was adopted.

4.5.1 The Chemical System and Stoichiometry

As described earlier[37,125-126] bromazepam forms a soluble purple complex with iron(II) in hydrochloric acid medium that absorbs at a maximum wavelength of 585 nm. The chelation of iron(II) was believed to be through a conjugated α,α'–diimine grouping, yielding a stable five-membered ring complex ion. The intense absorption of iron(II)-bromazepam complex ion is believed to be due to charge transfere from iron (II) to bromazepam ligand, in a similar fashion as in the case of iron(II)-1,10 phenanthroline complex ion.[127] The stoichiometry of this reaction was found to be 1:1 bromazepam to iron(II) mole ratio respectively under the specific conditions for the determination of the drug at a fixed time of 300.0 seconds and limited concentration range of both reactants in 0.02 mol dm^{-3} HCl. A thorough investigation of the stoichiometry and

behaviour of the reaction was found to be necessary to be studied under wider range of the reactants concentrations with absorbance measurements taken after the reaction reached completion. The continuous variation method[45](Job's plots) was probed and the curves were plotted after 24 hours. The results revealed that the complex formation at the two wavelengthes is more prominant resulting in higher absorbance values at lower than 0.0120 mol dm^{-3} HCL indicating that the stoichiometry is acid dependent. This could be attributed to the competing ability of the drug with hydrogen ions to the coordinated sites of iron(II) octahedral central ion. This complexation trend is quite similar to that of iron(II) with 1:10-phenanthroline[128] which is a quite familiar one. However, the existence of both types of complexes 1:1 and 3:1 at the same time could not be denied since it is not feasible to demark the acidity of formation of one complex from the other. It is interesting to note that the 3 to 1 mole ratio complex appears at a maximum wavelength of 585 nm. This bathochromic shift to a longer wavelength is indicative of greater stability of the compound that could be explained by a charged ion surrounded by three molecules of bidentate character.

In figure 4.17 spectra were recorded at different times for the mixture of 3.190×10^{-2} mol dm^{-3} iron(II) and 3.660×10^{-4} mol dm^{-3} bromazepam in 8.4000×10^{-3} mol dm^{-3} HCl. Two peak maxima were observed at 585 and 525 nm for the 3 : 1 and 1 : 1 complexes respectively. It is clear that the formation of both complexes is slow and that the formation of the 1 : 1 complex at 525 nm starts first and later the peak maximum for the 3 : 1 complex at 585 nm appears. It is also clear that the rate of formation of the

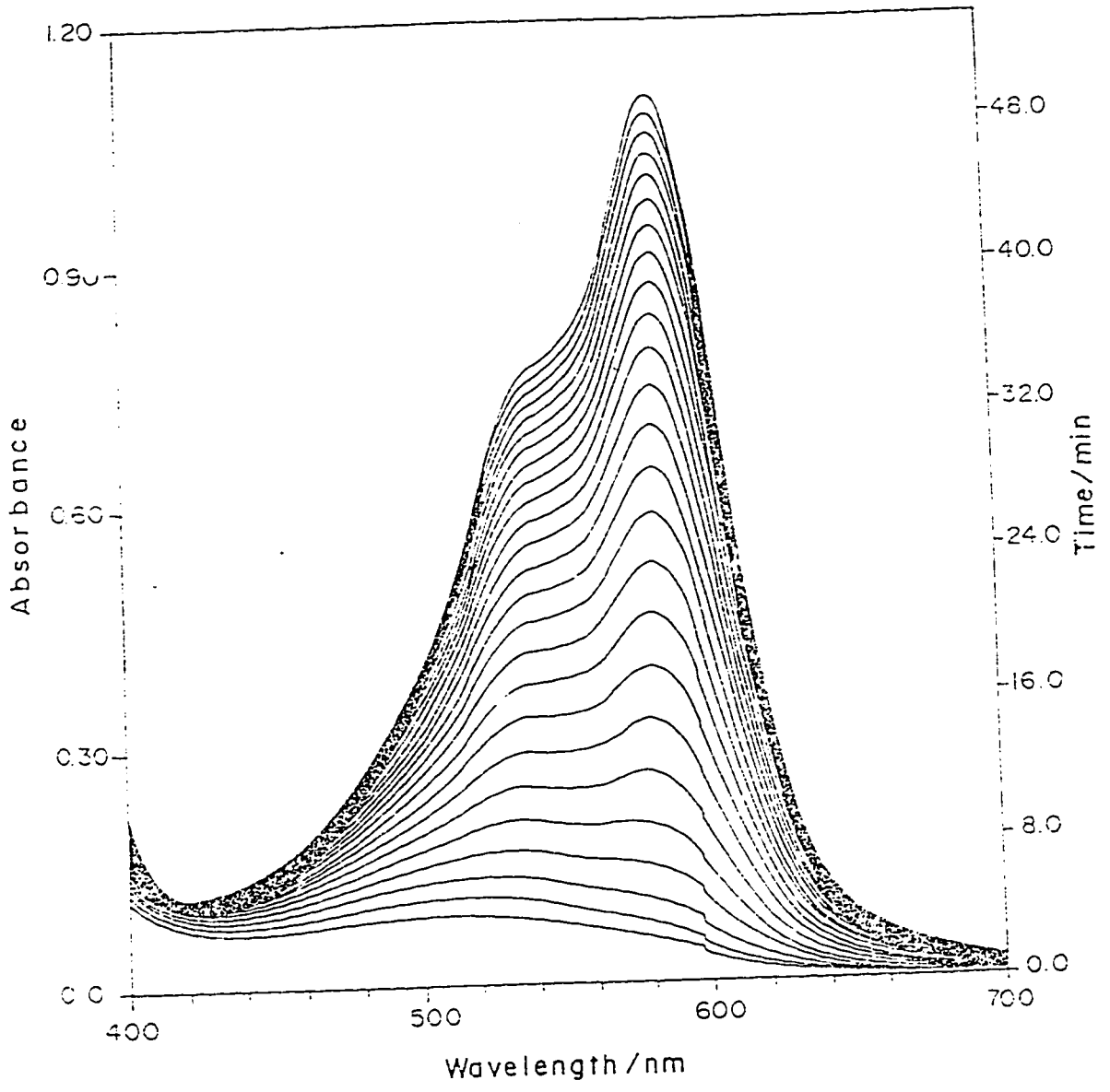


Figure 4 . 17Tracing of the spectra recorded at different times for the complexation of iron(II) with bromazepam. Conditions: [iron(II)] = 3.1900 x 10^{-2} mol dm^{-3}; [BRZ] = 3.6600 x 10^{-4} mol dm^{-3}; [HCl] = 8.4000 x 10^{-3} mol dm^{-3}.

3 : 1 complex is faster than the other, thereby resulting in equal peak absorbances at ten minutes and higher peak absorbance of the 3 : 1 complex later on.

4.5.2 Reactions Kinetics

The complexation reaction was found to be reasonably slow in hydrochloric acid concentration range of 0.0100 to 0.0400 mol dm^{-3} allowing for possible kinetic investigations to be followed spectrophotometrically. Figure 4.18 shows a typical kinetic curve generated by aspirating 3 s of 4.58×10^{-3} M bromazepam and 3 s of 5.00×10^{-3} M iron(II), both reagents are prepared in 0.0115 M HCl, into a carrier solution of 0.0115 M HCl. From this figure it is obvious that the investigation of the kinetics of this system using SI-technique is quite feasible.

The partial reaction orders with respect to the different variables assumed to have influence on the rate equation was carried out by considering the differential form of the rate equation involving the pseudo zero-order reactions; when the rate of formation of the products are virtually negligible. In this method all reactants, except the one under investigation [A], are kept constant at higher concentrations and measuring rates when that reactant concentrations are varied. Under such conditions, the following equation applies :

$$rate(\nu) = \frac{d[P]}{dt} = k'[A]^n \qquad 4.8$$

Where :

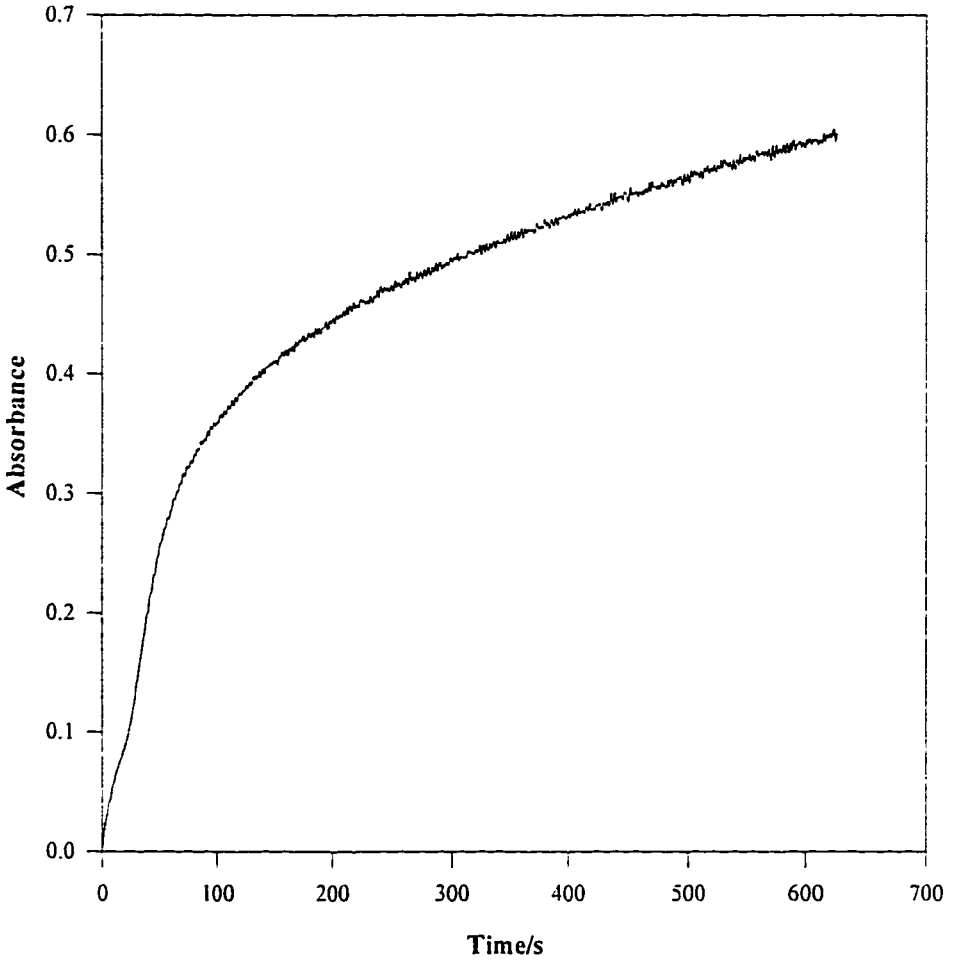


Figure 4.18 Typical SI-kinetics curve; [iron(II)] = 5.00×10^{-3} M [BRZ] = 4.85×10^{-3} M; $[H_2SO_4]$ = 0.0115 M; delay time (t_d) = 12 s; stop time (t_s) = 600 s.

P is the reaction product i.e. the complex in this study.

k' is the pseudo-nth-order rate constant

n is the partial reaction order.

Taking logarithm of equation (4.8) :

$$\log v = \log k' + n \log[A] \qquad 4.9$$

From the slope of the above equation, the partial reaction order could be obtained.

4.5.2.1 Reaction Order with Respect to [H^+]

It was observed that the reaction rate decreases as the acid concentration is increased as represented by figure 4.19 which was recorded for [iron(II)] = [BRZ] = 4.58 X 10^{-3} mol dm^{-3} while hydrochloric acid concentration was varied between 0.0115 mol dm^{-3} to 0.040 mol dm^{-3}.

Rates were calculated from the plot by applying the fixed-time method[17,18] involving measuring the absorbance as (ΔA) of the product at a predetermined time (Δt)from the start of the reaction. The plot of $\log(\Delta A/\Delta t)$ versus log [HCl] resulted in a straight line the slope of which was -0.953 with a correlation coefficient (R^2) of 0.977. The order of the reaction with respect to hydrogen ion concentration is therefore equal to inverse one (-1), indicating that hydrogen ions are generated as a product.

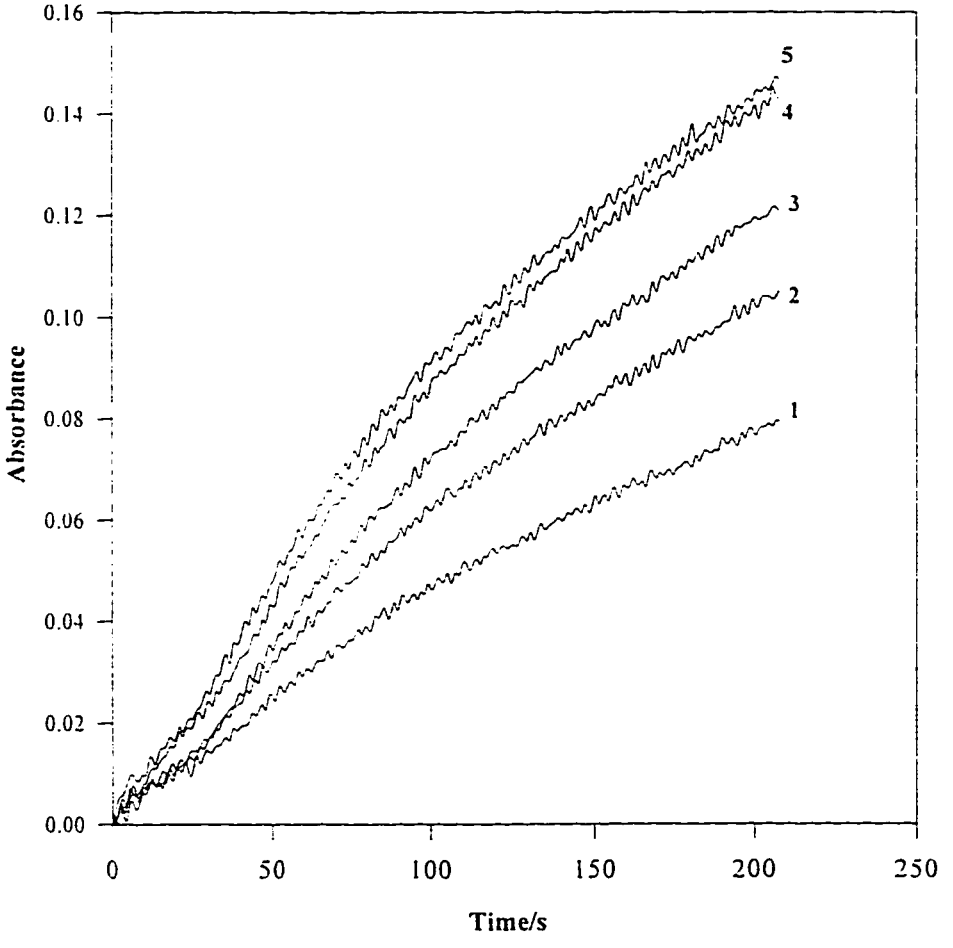


Figure 4.19 Absorbance time curves for the determination of the order of the reaction with respect to hydrogen ions. [iron(II)] = [BRZ] = 4.5800 x 10^{-3} mol dm^{-3}; [HCl] = (1) 0.0400; (2) 0.0300; (3) 0.0200; (4) 0.0150; (5) 0.0115 mol dm^{-3}; delay time (t_d) =12.0 s; stopped flow time (t_s) = 200 s.

4.5.2.2 Reaction Order with Respect to [Iron(II)]

Figure 4.20 shows absorbance-time curves for fixed concentrations of 0.0115 mol dm^{-3} acid, 3.7800 X 10^{-3} mol dm^{-3} bromazepam for different concentrations of iron(II) varied between 4.58 X 10^{-5} mol dm^{-3} to 3.20 X 10^{-4} mol dm^{-3}. The regression analysis of the plot of $\log(\Delta A/\Delta t)$ versus log [iron(II)] gave a straight line, with a slope of 1.09 and a correlation coefficient (R^2) of 0.987. Therefore the reaction order with respect to iron(II) is equivalent to one.

4.5.2.3 Reaction Order with Respect to [BRZ]

Figure 4.21 shows absorbance-time curves plotted at constant concentrations of 0.0573 mol dm^{-3} iron(II),0.020 mol dm^{-3} acid taking different concentrations of bromazepam varying between 2.75 X 10^{-4} mol dm^{-3} to 5.50 X 10^{-4} mol dm^{-3}. The plot of $\log(\Delta A/\Delta t)$ versus log [BRZ] gave a straight line with a slope of 1.03 and a correlation coefficient (R^2) of 0.993 indicating that the order of reaction with respect to bromazepam is one.

4.5.2.4 Activation Energy

Experiments were conducted at temperatures varied between 25.0-50.0 ^{o}C using equimolar solutions of iron(II) and bromazepam of 4.85 $\times 10^{-3}$M and by maintaining the acid concentration to 0.0115 M. The pseudo-second order rate constants were calculated for each kinetics curve

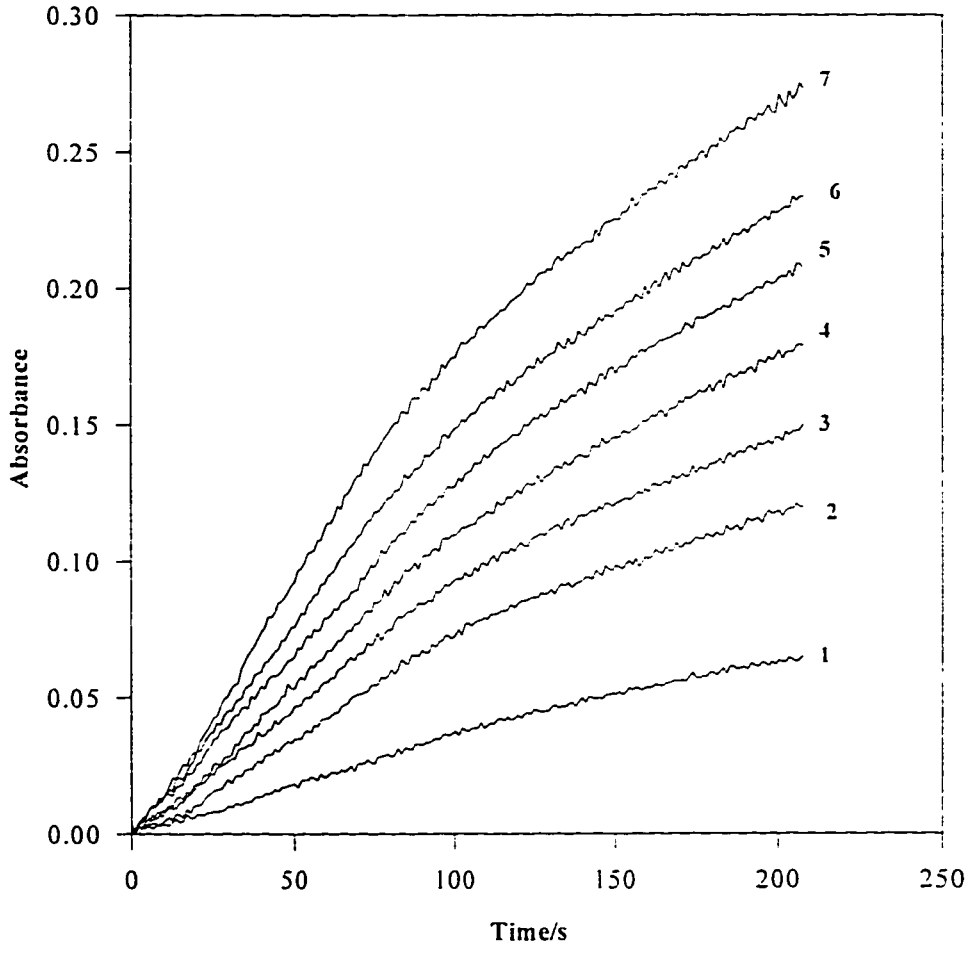


Figure 4.20 Absorbance time curves for the determination of the order of the reaction with respect to iron(II). [BRZ] = 3.7800 x 10^{-3} mol dm^{-3}; [HCl] = 0.01150 mol dm^{-3}; [iron(II)] = (1) 4.5800 x 10^{-5}; (2) 9.1600 x 10^{-5}; (3) 1.3700 x 10^{-4}; (4) 1.8300 x 10^{-4}; (5) 2.2900 x 10^{-4} (6) 2.7500 x 10^{-4} ; (7) 3.2000 x 10^{-4} mol dm^{-3} ; delay time (t_d) =12.0 s; stopped flow time (t_s) = 200 s.

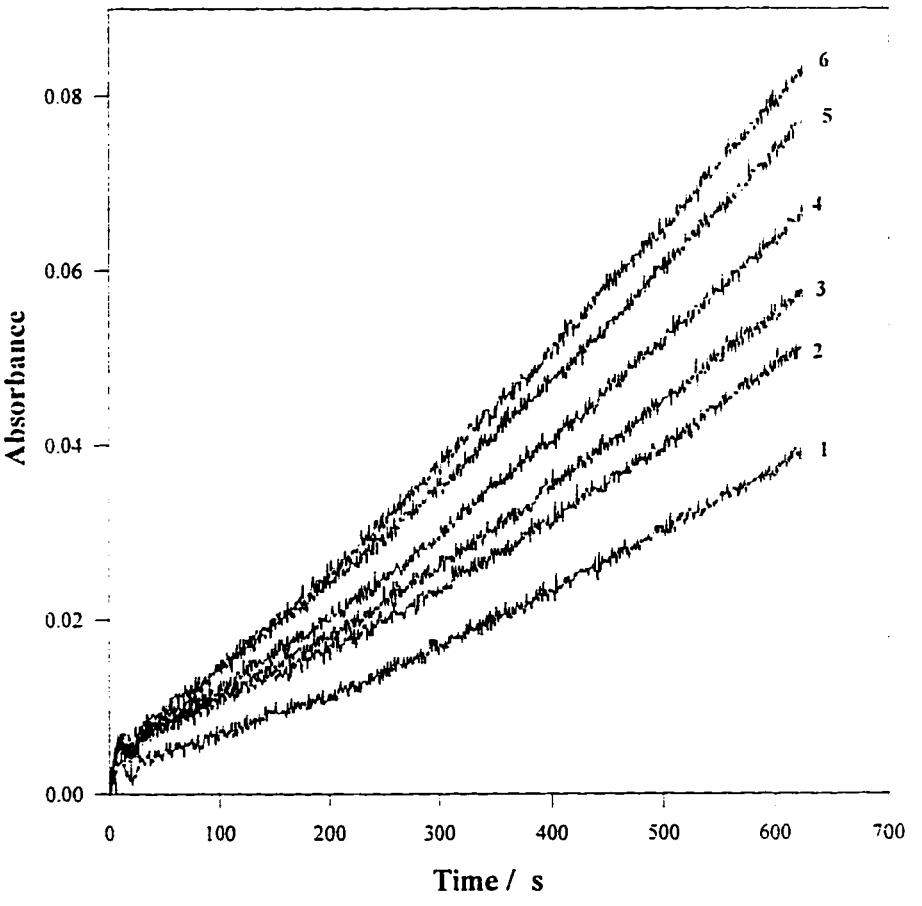


Figure 4.21 Absorbance time curves for the determination of the order of the reaction with respect to bromazepam. [iron(II)] = 5.7300×10^{-2} ; [HCl] = 0.0200 mol dm^{-3}; [BRZ] = (1) 2.7500×10^{-4}; (2) 3.6600×10^{4}; (3) 4.1200×10^{-4}; (4) 4.5800×10^{-4}; (5) 5.0400×10^{-4} (6) 5.5000×10^{-4} mol dm^{-3}; delay time (t_d) = 12.0 s; stopped flow time (t_s) = 600 s.

at different temperatures and are given in table (4.13). Regression analysis of the plot of $\log k'$ versus 1/T was carried out using the following Arrhenius(R) equation:

$$\log k' = \frac{-E_a}{2.303R} \cdot \frac{1}{T} + \log[A] \qquad 4.10$$

where Ea is the activation energy; R is the gas constant (R = 8.314 J K^{-1} mol^{-1}); T is the temperature in Kelvin and A is a pre-exponential factor. The activation energy was calculated from the slope of the previous plot and was found to be 41.7 kJ mol^{-1}.

4.5.3 Reaction Mechanism

The phenomenon that reaction rate accelerates as the acid concentration decreases and slows down as the acid concentration increases indicates that the deprotonated form of the bromazepam is the active complexing species and that the reaction starts once a deprotonation equilibrium step takes place before the rate-determining step as follows :

$$BRZ\text{-}N_2H_2^{2+} \longleftrightarrow BRZ\text{-}N_2H^{1+} + H^+ \qquad 4.11$$

It is important to note that three pK values were reported for the bromazepam[125] at 2.5, 5.2 and 11.8 attributed to the protonation at the adjacent azomethine and pyridine nitrogen atoms and the deprotonation at the other nitrogen atom of the azomethine group respectively. In

Table 4.13 Calculated values for rate constants for reaction mixtures containing 4.58 x 10^{-4} mol dm^{-3} iron(II), 4.58 x10^{-4} mol dm^{-3} bromazepam and 0.0115 mol dm^{-3} HCl.

t(^{0}C)	1/T (K)	k', x 10^3	Log k'
28.0	3.32	0.758	-3.12
35.0	3.25	1.162	-2.93 ~~-3.93~~
40.0	3.19	1.479	-2.83
45.0	3.14	1.812	-2.74
50.0	3.09	2.315	-2.64

hydrochloric acid concentration range of 0.0115 mol dm^{-3} to 0.040 mol dm^3, of the present study, bromazepam will be present as $BRZ\text{-}N_2H^{1+}$ thereby acting as the active species initiating the complexation reaction represented by equation (4.13).

From the kinetics observed the rate law could be deduced by the following equation :

$$\frac{d[(BRZ-N_2)_3 Fe^{2+}]}{dt} = k[Fe^{2+}][BRZ\text{-}N_2H_2^{2+}][H^+]^{-1} \qquad 4.12$$

Hence the following mechanism was proposed to be consistent with the rate law in equation (4.12).

$$BRZ\text{-}N_2H^{1+} + Fe^{2+} \longrightarrow (BRZ\text{-}N_2)Fe^{2+} + H^+ \qquad 4.13$$

$$BRZ\text{-}N_2H^{1+} + (BRZ\text{-}N_2)Fe^{2+} \longrightarrow (BRZ\text{-}N_2)_2Fe^{2+} + H^+ \qquad 4.14$$

$$BRZ\text{-}N_2H^{1+} + (BRZ\text{-}N_2)_2Fe^{2+} \longrightarrow (BRZ\text{-}N_2)_3Fe^{2+} + H^+ \qquad 4.15$$

The reaction represented by equation (4.13) for the formation of the 1 : 1 complex, which was proven above to be the slowest, is considered the rate determining step. The mechanism clearly explains that higher acid

concentration favors the existence of lower drug to iron(II) mole ratio while lower acid concentrations favors higher ratios as indicated by reactions in equations (4.14) and (4.15) following the rate determining step.

4.5.4 The Kinetic Method

The fixed-time method[17,18] was found to be suitable for the determination of bromazepam. From the kinetic data, 0.0100 mol dm^{-3} iron(II) in 0.0115 mol dm^{-3} hydrochloric acid concentrations were taken fixed for the determination of the bromazepam at different fixed times of 50.0, 75.0, 100.0 and 150.0 seconds. Based on the correlation coefficient (r^2) and the intercept (βo), of the regression of the bromazepam concentration versus absorbance of the complex (Table 4.14), the fixed time of 100.0 second was found to be suitable for taking measurements. Therefore, the analysis volume (V_A) was aspirated for exactly 100 seconds where it reaches the detector for absorbance measurements. Regression plots were found to be linear for bromazepam concentration in the range 5.0 x 10^{-4} to 1.5 X 10^{-3} mol dm^{-3}.

4.5.5 Application

The sequential injection fixed-time method was applied to the determination of bromazepam in the proprietary drug lexotanil tablets. The analysis was repeated six times and the results obtained were found to be highly precise with a relative standard deviation of less than 1.2% and a

Table 4.14 Calibration equations obtained at different fixed times taking constant concentrations of 0.010 mol dm^{-3} iron(II), 0.0115 mol dm^{-3} HCl and variable bromazepam concentrations ranging between 5.0 x 10^{-4} to 1.3 x 10^{-3} mol dm^{-3}.

time (s)	calibration equation	r^2
50	A = -0.1142 + 181.33 C	0.991
75	A = -0.1329 + 217.4 C	0.994
100	A = -0.149 + 247.7 C	0.996
150	A = -0.149 + 285.17 C	0.996

recovery of not less than 99.89% of the claimed content in the tablet. The method suffered no interferences from excipients usually added to the tablet formulations such as glucose, manitol, sucrose starch etc. The accuracy was judged by a statistical comparison of the results of the analysis obtained for the same batch of samples with those obtained by the FIA method.[37] The student t-test values indicated no significant differences between the two methods rendering our method a highly accurate and a reliable one.

The sequential injection fixed-time method is simple, accurate, more sensitive than the spectrophotometric methods[129-133] and the electrochemical methods[134-135] of this compound. The method is not elaborate and time consuming as the chromatographic methods reported[136-144].

CHAPTER FIVE

5. CONCLUSION

In this study a systematic application of chemometric methods of optimization was performed in an attempt to develop and validate analytical methods suitable for the assay of some selected drugs to be applied in their pharmaceutical preparations. The methods developed, were aimed to satisfy some desirable features required for routine analysis. These features include, simplicity, short time of analysis where no sacrifice should be made with respect to precision and accuracy. Tolerance of the methods to uncontrolled factors, known as ruggedness, is also considered. The search technique employed was the super modified simplex technique.

The recently developed FI-technique and its newly born sister SI-technique were undertaken, and the full utilization of these technique was a prime goal to be achieved and demonstrated during this work.

In the determination of promethazine and ciprofloxacin super modified simplex program was successfully used for the optimization of four independent variables; reaction coil length, flow rate, cerium(IV) concentration and sulfuric acid concentration. For both systems the optimum operating conditions were reached faster with few number of

experiments. In the promethazine system the sensitivity was selected as the primary performance criterion whereas the time of analysis was the secondary criterion. The secondary criterion was maintained by setting the lower threshold of the flow rate to 4.10 ml/min to obtain a sampling frequency of not less than 120 h^{-1}. The method developed was applied for the determination of promethazine in drug formulations. This method was found to be superior to official methods with a wide dynamic range (60-200 ppm) and with an excellent reproducibility. For the ciprofloxacin system, a composite performance criterion was used in which the sensitivity and the analysis time were formulated in one expression. The method developed for the assay of this drug was based on the complexation of the drug on line using iron(III) and monitoring the resulting product at 447 nm. This method was the first of its kind in literature for the determination of this new drug in pharmaceutical products. A wide dynamic range of 50-500 ppm was attained with a relative standard deviation of less than 0.96%.

Statistical experimental design in conjunction with the super modified simplex technique was used in order to optimize analytical systems with respect to sensitivity and time of analysis. A 2^5 full factorial design, where 5 indicates the number of variables and 2 represents the higher and lower levels of these variables, was used prior to the simplex method for optimization of operating conditions for the assay of procainamide-hydrochloride and norfloxacin antibiotic. In the procainamide system two factors were excluded from the post-simplex optimization as inferred from the factorial design study priorly performed.

The factors excluded from the simplex optimization were the flow rate and sample loop size. The method developed for the assay of procainamide-hydrochloride in pharmaceutical preparations was characterized by a wide dynamic range of 100-600 ppm. A high degree of accuracy was demonstrated by the method together with an excellent sampling frequency of 250 h^{-1}. In the norfloxacin system only the coil length was found to be insignificant from the factorial design screening results. The other four factors; iron(III) concentration, flow rate, sulfuric acid concentration and sample loop size were then included in the simplex optimization. The optimum operating conditions were obtained after 22-experiments. The proposed method for norfloxacin was characterized by a wide dynamic range of 50-450 ppm, excellent reproducibility and a high sampling frequency of at least 140 h^{-1}.

A different strategy was adopted for the determination of the optimum operating conditions for the case of chlorpromazine and perphenazine. At first, a simplex method was applied to attain optimum operating conditions which were further confirmed by response surface methodology (RSM). Application of properly designed experiments lead to better understanding of the response surface and safer prediction of optimum conditions. In both systems the phenothiazine drug was oxidized on line with cerium(IV) in sulfuric acid media generating a radical product which was the species monitored spectrophotmetrically. In chlorpromazine system only the chemical variables were included in an attempt to correlate the behavior of these factors to the kinetics of the

reaction. A more comprehensive study was performed in the case of perphenazine system, where five variables were involved as well as a composite performance criterion where sensitivity and analysis time were combined in one expression.

Response surface methodology (RSM) has enabled modeling the response surface and was used successfully to correlate the experimental variables to the corresponding response. Empirical models were built, and it was found that second order polynomials with interactions were quite satisfactory to describe the surfaces studied. Such models provided useful multidimensional information about the systems investigated and also assisted in the optimization. On the other hand multidimensional visualization deemed to be necessary and provided highly informative illustrations. In this study only two-dimensional surface plots were generated because they are easy to understand. Other multidimensional contour plot were found very difficult to understand and to visualize.

In the second part of this study sequential injection (SI) technique was applied to the determination of concentrations, stoichiometries, and formation constants of complexation reactions. Also the versatility of the technique was examined for optimization as well as for the kinetic studies involving the development of a kinetic method of analysis.

The capacity of the SI-technique was first demonstrated by applying the technique, for the first time, to the determination of stoichiometries of

complexation reactions. The first system studied was the complexation of perphenazine and trimeprazine with palladium(II) in dilute hydrochloric acid media. The Job's method of continuous variation was utilized and the drug : palladium(II) mole ratio was found to be 1 : 1 for both systems. It is interesting to note that both reagents, the drug and palladium(II), were aspirated in μl amounts, thus consuming very small amount of the reagents. This is a great advantage achieved, making feasible the full investigation of physico-chemical parameters which are usually ignored or studied superficially to avoid consuming a valuable and expensive reagent. This study opens the way for scientists from discipline other than those involved in the chemical analysis to use such a technique.

The second system investigated here was the complexation of ciprofloxacin and norfloxacin with iron(III). For this system the determination of formation constants and stoichiometries was probed by SI-technique and making use of advanced numerical methods of analysis. Two programs were used; the Jobcon program and the Merlet program, to manipulate the continuous variation data and the molar ratio data. A large number of experimental trials was performed utilizing a relatively small amount of reagents. The values obtained for the formation constants for both systems were found to be closely related of literature values.

Optimization studies were found to be easier when SI-technique is used compared to the conventional flow injection (FI) technique, owing to the simple mechanical assembly of the former. A factorial design, where

more than three levels were involved, was used for the development of a method for the assay of ciprofloxacin by SI-technique. The all-model model search technique was utilized to develop the best model that can describe the response surface and to obtain the optimum conditions for the assay of these drugs. This procedure was found easy and robust and enabled establishing confident optimum conditions for the economic assay of ciprofloxacin and norfloxacin by SI-technique.

In the oxprenolol system an orthogonal array design was applied for the optimization of the experimental variable for the assay of this drug. The method developed was based on the oxidation of the drug with cerium(IV) in sulfuric acid concentration.

Finally, the SI-technique was utilized to fully investigating the kinetics of the complexation of bromazepam with iron(II) in hydrochloric acid media. The reaction orders with respect to each reagent were determined by SI-technique and were found to be 1, 1, and -1 for bromazepam, iron(II) and hydrogen ions respectively. On the basis of these values a rate law was developed and a plausible mechanism was established. A kinetic method for the analysis of bromazepam in drug formulations was also developed based on the results obtained above. This full investigation of the kinetics of the reaction using SI-technique was the first of its kind in the literature.

As was observed from the literature, most of the kinetic methods of analysis reported lack a full investigation of the kinetics and mechanism of the chemical reactions involved. Therefore, it can be seen that with the emergence of robust techniques such as the SI-technique there is a excellent chance to reshape the kinetic methods of analysis in order to include a full investigation of the kinetics and mechanism of the chemical reaction. In addition the computer controlled SI-technique could lead to a vast resurgence in the activity in this field and other related ones.

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APPENDIX A

JOBCON PROGRAM PSEUDOCODE

```
type
 mn=record
  integer m,n
  real AM,YMA,XM,LogKP,P1,P2,P3
 end
 min=record
  integer m,n
  real value
 end

min best
mn array (systems_{i,j})_{1:8,1:8}

real function YMI(integer m,integer n,real X,real A,real AM)
 real E1,E2,YM2,Y
 real C1,C2,C3,F,FP,temp,YM1,Accuracy
 YM2←0.5
 Y←A/AM
 YM1←0.001
 Accuracy ← 0.000000001
 E1 ← (m+n)X-mY
 E2 ← (m+n)(1-X)-nY
```

```
C1 ← (E1^m)(E2^n)
C2 ← Y(n^n)(m^m)
C3 ← m+n
if ((E1)(E2)>0) then
  while (| YM2-YM1| >=Accuracy) do
    F ← C1 - C2 (1-YM1)^C3/YM1
    FP ← C2(1-YM1)^C3-1(YM1(C3-1)+1)/YM1^2
    YM2 ← YM1-F/FP
    temp ← YM2
    YM2 ← YM1
    YM1 ← temp
  end do
  YMI←YM2
 else
  YMI←0.0000001
 end if
end function YMI

real function MAY(integer m,integer n,real AM)
 real Sum
 Sum ← 0
 for i=1 to L do
  YM_i ← YMI(m,n,X_i,A_i,AM)
  Sum←Sum+YM_i
 end do
 MAY←Sum/L
end function MAY

real function P1(integer m,integer n,real AM)
```

```
 real YMA,D1,Sum
 Sum ← 0
 YMA ← MAY(m,n,AM)
 for i=1 to L do
   D1 ← YMA - YM_i
   Sum ← Sum+D1^2
 end do
 P1=√(Sum/(L-1))
end function P1

real function P2(integer m, integer n,real AM)
 real YMA,D2,Sum
 Sum ← 0
 YMA ← MAY(m,n,AM)
 for i=1 to L do
   D2 ← (YMA - YM_i)/YMA
   Sum ← Sum + D2^2
 end do
 P2←√(Sum/(L-1))
end function P2

real function P3(integer m,integer n,real AM)
 real YMA,D3,Sum
 Sum ← 0
 YMA ← MAY(m,n,AM)
 for i=1 to L do
   D3 ← (YMA - YM_i)/ YMA^2
   Sum ← Sum + D3^2
 end do
```

```
  P3 ← (Sum/(L-1))
end function P3

real function minimum(integer m,integer n,real (f)(integer,integer,real),real a, real b)
  real x,y,u,v,r,Accuracy
  r ←0.5(√(5)-1)
  Accuracy ← 1e-15
  x ← a + r(b-a)
  y ← a + r²(b-a)
  u ← f(m,n,x)
  v ← f(m,n,y)
  while ( (x-y)>Accuracy )
    if (u>v)
      b ← x
      x ← y
      u ← v
      y ← a + r²(b-a)
      v ← f(m,n,y)
    else
      a ← y
      y ← x
      v ← u
      x ← a + r(b-a)
      u ← f(m,n,x)
    end if
  end do
  minimum ← x
end function minimum
```

```
real function LogK(integer m,integer n.real YMA)
 LogK ← log10((m+n)/C)^(m+n-1))YMA/m^m n^n(1-YMA)^(m+n))
end LogK

procedure ReadData
 read(C)
 read(P)
 read(L)
 for i = 1 to L do
  read(X_i,Ai)
 end do
end procedure ReadData

procedure ApplyAndOutput(integer m,integer n, real min, real max)
 systems_m,n m← m
 systems_m,n n←n
 systems_m,n AM← minimum(m,n,P3,min,max)
 systems_m,n YMA← MAY(m,n,systems_m,n .AM)
 systems_m,n P1← P1(m,n,systems_m,n AM)
 systems_m,n P2← P2(m,n,systems_m,n AM)
 systems_m,n P3← P3(m,n,systems_m,n AM)
 systems_m,n XM← m/(m+n)
 systems_m,n LogKP← LogK(m,n,systems_m,n YMA)
 if (systems_m,n P2<best.value)
  best.value = systems_m,n P2
  best.m ← m
  best.n ← n
 end if
 output(
```

```
    m,
    n,
    systems_{m,n}AM,
    systems_{m,n}XM,
    systems_{m,n}YMA,
    systems_{m,n}LogKP,
    100systems_{m,n}P2  )
end procedure ApplyAndOutput

program main
 integer  L,m,n
 real (YM_i)_100,(X_i)_100,(XM_i)_100,(A_i)_100,C,P
 real min,max
 min←0.0001
 max←10.00
 best.value←100.0
 ReadData
 output("m : n   AM       XM       YMA      Log K'     RE")
 for n=1 to 8 do
  call ApplyAndOutput(1,n,min,max)
 end do
 for m=2 to 8 do
  call ApplyAndOutput(m,1,min,max)
 end do
 output("These values are best suited :")
  call ApplyAndOutput(best.m,best.n,min,max)
end program main
```

APPENDIX B

PSEUDOCODE FOR MERLET PROGRAM

```
real function f(real x,real K,integer i,integer m,integer n)
   real clbar,term1,a,b,c,d,e,term2,qm
   clbar←cl/fl,
   term1 ←x*fl*m/n
   a←x^(1/m)
   b←n*K^(1.0/m)
   c←clbar^((m+n-1.0)/m)
   d←fl^((n-1.0)/m)
   e←|1-x|^(1*m/n)
   term2←a/(b*c*d*e)
   qm ←Vo/V_t
   f ←term1+term2-qm
end function f

real function root(real (*f)(real,real,integer,integer,integer),real a,real b,real
        K,integer i,integer m,integer n)
  real temp,fa,fb
  integer no_of_iterations
  no_of_iterations←0
  fa←f(a,K,i,m.n)
  fb←f(b,K,i,m,n)
  while ( |fa|>=0.0001) and (no_of_iterations<100) ) do
    no_of_iterations←no_of_iterations +1
```

```
  if (|fa|>|fb|)
    temp←a
    a←b
    b←temp
    temp←fa
    fa←fb
    fb←temp
  end if
    temp←b-a)/(fb-fa)
    b←a
    fb←fa
    a←a-fa*temp
    fa←f(a,K,i,m,n)
  end do
  if (no_of_iterations>=100) then
    output("Iterations Exceeded Limit at point :",i)
  end if
root←a
end function root


procedure initial_alpha_test(real Amax)
  for i=1 to L do
    Alpha_i←A_i*(1+V_i/Vo)-Ao)/(Amax-Ao)
    if ( (|Alpha_i|<0) or (|Alpha_i|>1) ) then
      output("Alpha out of range for point #",i)
      output("point discarded")
      for j=i+1 to L do
          V_j-1←V_j
```

```
        A_{j-1} ←Aj
      end do
      L←L-1
      I←I-1
     end if
   end do
   Acalc_1←A_1
   Acalc_2←A_2
   for I=3 to L do
    Acalc_i←(1+V_i/Vo)A_i
   end do
end procedure initial_aplha_test

real function U(real Amax,real K,integer m,integer n)
   real root,cl,absorbance_kaba,root_kaba,Sum
   Sum←0
   for I=1 to L do
     absorbance_kaba ←(A_i-Ao)/(Amax-Ao)
     root_kaba←root(f,0.00001,0.99999, K, i, m, n)
     Sum←Sum+(absorbance_kaba-root_kaba)^2
   end do
   U ←1/Sum
end function U

procedure readdata
   read(cmo)
   read(cl)
   read(Ao)
   read(Vo)
```

```
 read(L)
 read(m,n)
 for i=1 to L do
  read(V_i)
  read(A_i)
 end do
end procedure readdata

program main
 integer m,n
 real (V_i)_1:100,(A_i)_1:100,(Alpha_i)_1:100,(Acalc_i)_1:100
 real cl,Ao,Vo,L,cmo,ccl,K,Amax,fl=1
 call readdata
 read(m,n)
 read(Amax,K)
 call initial_alpha_test(Amax)
 output (U(Amax,K,m ,n))
end program main
```